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RESEARCH PAPER

Gonadal hormone-independent sex differences in $GABA_A$ receptor activation in rat embryonic hypothalamic neurons

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Background and Purpose: $GABA_A$ receptor functions are dependent on subunit composition, and, through their activation, GABA can exert trophic actions in immature neurons. Although several sex differences in GABA-mediated responses are known to be dependent on gonadal hormones, few studies have dealt with sex differences detected before the critical period of brain masculinisation. In this study, we assessed $GABA_A$ receptor functionality in sexually segregated neurons before brain hormonal masculinisation.

Experimental Approach: Ventromedial hypothalamic neurons were obtained from embryonic day 16 rat brains and grown in vitro for 2 days. Calcium imaging and electrophysiology recordings were carried out to assess $GABA_A$ receptor functional parameters.

Key Results: $GABA_A$ receptor activation elicited calcium entry in immature hypothalamic neurons mainly through L-type voltage-dependent calcium channels. Nifedipine blocked calcium entry more efficiently in male than in female neurons. There were more male than female neurons responding to GABA, and they needed more time to return to resting levels. Pharmacological characterisation revealed that propofol enhanced $GABA_A$-mediated currents and blunted GABA-mediated calcium entry more efficiently in female neurons than in males. Testosterone treatment did not erase such sex differences. These data suggest sex differences in the expression of $GABA_A$ receptor subtypes.

Conclusion and Implications: GABA-mediated responses are sexually dimorphic even in the absence of gonadal hormone influence, suggesting genetically biased differences. These results highlight the importance of $GABA_A$ receptors in hypothalamic neurons even before hormonal masculinisation of the brain.

Abbreviations: PN, post-natal day; DIV, days in vitro; E, embryonic day; E_{GABA}, GABA-evoked current; RMP, resting membrane potential; THIP, gaboxadol, 2H,3H,4H,5H,6H,7H-[1,2]oxazolo[5,4-c]pyridin-3-one; VDCC, voltage-dependent Ca^{2+} channels.

1 | INTRODUCTION

Sexual differentiation of the brain is mediated by gonadal hormones during embryonic development, within a timeframe called the "critical period," established between embryonic day 18 (E18) and post-natal day 10 (PN10) (McCarthy, Wright, & Schwarz, 2009). In this regard, the hypothalamus is the most sexually dimorphic region of the brain. During development and adult life, the hypothalamus is highly sensitive to sexual steroids that mediate sexual differentiation, due to its elevated levels of gonadal hormone receptors (Lenz & McCarthy, 2010). In addition, genetic information encoded in sex chromosomes is critical for hypothalamic sexual differentiation (Büdefeld, Tobet, & Majdič, 2010; Grgurevic, Büdefeld, Spanic, Tobet, & Majdic, 2012; Majdic & Tobet, 2011).

During development, neural communication by autocrine and paracrine signals is fundamental for growth and connectivity. In this regard, GABA is the main excitatory neurotransmitter released by neurons during embryonic development (Ben-Ari, 2002). **$GABA_A$ receptors** are Cl^- channels composed of five subunits arranged around a central pore. In mammals, 19 subunits have been characterised (6 α, 3 β, 3 γ, 1 δ, 1 ε, 1 π, 1 θ, and 3 ρ subunits) (Simon, Wakimoto, Fujita, Lalande, & Barnard, 2004), the expression of which depends on the hypothalamic developmental stage (Laurie, Wisden, & Seeburg, 1992; Pape et al., 2009). Subunit composition alters the pharmacological and electrophysiological properties of $GABA_A$ receptors, an effect critical for the physiology, intrinsic activity, affinity, and efficacy of several $GABA_A$ receptor modulators (Johnston, 1996; Korpi, Gründer, & Lüddens, 2002; Olsen & Sieghart, 2008).

Particularly, immature neurons display high intracellular Cl^- concentration ($[Cl^-]_i$), which generates a reversal potential of GABA-evoked current (E_{GABA}) that is positive to resting membrane potential (RMP). Therefore, activation of $GABA_A$ receptors exerts depolarising effects on immature neurons, triggering action potentials (Wang, Gao, & van den Pol, 2001) and Ca^{2+} influx by gating **voltage-dependent Ca^{2+} channels (VDCC**; Obrietan & van den Pol, 1995). The excitatory effects of $GABA_A$ receptors are needed for neuronal development as disruption of Cl^- gradients in immature neurons would markedly affect neuronal maturation (Cancedda, Fiumelli, Chen, & Poo, 2007; Reynolds et al., 2008) and synaptic connectivity (Chudotvorova et al., 2005).

Nevertheless, the excitatory actions of $GABA_A$ receptors switch to hyperpolarising effects (characteristic of mature and adult neurons) during early post-natal stages. At this time, gonadal hormones mediate brain sexual differentiation (McCarthy et al., 2009). The hypothalamus displays marked sex differences in several GABAergic parameters, including ion current kinetics (Smith, Brennan, Clark, & Henderson, 1996), GABA levels (Davis, Ward, Selmanoff, Herbison, & McCarthy, 1999), response to diazepam (Kellogg, Yao, & Pleger, 2000), excitatory versus inhibitory actions of GABA (Auger, Perrot-Sinal, & McCarthy, 2001), and levels of the Cl^- transporters, **NKCC1** and **KCC2** (Perrot-Sinal, Sinal, Reader, Speert, & McCarthy, 2007). There is evidence of a close relationship between GABA signalling and hormonally mediated sexual differentiation of the brain (McCarthy, Auger, & Perrot-Sinal, 2002; Perrot-Sinal, Davis, Gregerson, Kao, & McCarthy, 2001; Zhou, Pfaff, & Chen, 2005).

What is already known

- Sex hormones are responsible for the majority of GABA-mediated sex differences.

What this study adds

- Rat hypothalamic neurons exhibit hormone-independent sex differences in $GABA_A$ receptor activation and propofol modulation.

What is the clinical significance

- Male and female rats differ in $GABA_A$ receptor responses to clinically important drugs.

Previously, we have reported several hormone-independent sex differences in hypothalamic neurons during development (Cambiasso, Díaz, Cáceres, & Carrer, 1995; Cambiasso, Colombo, & Carrer, 2000), detected before the testosterone surge by embryonic testes (E18; Huhtaniemi, 1994). Moreover, we have found sex differences in gene expression of the neuritogenic transcription factor Ngn3 in E14 mice embryos, mostly relying on sex chromosomes (Scerbo et al., 2014). Regarding GABA, in cultured neurons (E16) after 9 days in vitro (DIV), we have detected a larger population of male than female neurons depolarising after $GABA_A$ receptor stimulation. Male neurons also displayed larger and longer lasting responses than females after $GABA_A$ receptor activation, even in the absence of hormone exposure (Mir, Carrer, & Cambiassso, 2017).

In the present work, we explored sex-dependent differences in GABA response in immature hypothalamic neurons. Using cell Ca^{2+} imaging recordings and patch-clamp measurements, we found differential responses to GABA between immature male and female hypothalamic neurons (2 DIV) that were cultured before hormone exposure and brain masculinisation. Our data suggest that these differences probably rely on sexual genetic backgrounds, independent of the sexual hormone environment.

2 | METHODS

2.1 | Cell cultures

All animal care and experimental protocols were in accordance with the National Institutes of Health (NIH) guidelines and were approved by the Institutional Care and Use Committees of INIMEC-CONICET-UNC (Córdoba, Argentina) and the Universidad de Concepción (Concepción, Chile). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and

with the recommendations made by the British Journal of Pharmacology.

Hypothalamic cultures of rat embryonic (E16) neurons were prepared, as previously described (Cambiasso et al., 2000). Briefly, the presence of a vaginal plug was designated as gestational day 1; then, on the morning of gestational day 16, pregnant Wistar rats (RRID:RGD_2312511, n = 23) were anaesthetised with CO_2 and killed by cervical dislocation. Fetuses were quickly removed under sterile conditions and separated by sex through identification of the spermatic artery on developing testes. After removing the brain, three to five ventromedial hypothalami of each sex were dissected out, pooled, and then incubated in trypsin-Hank's solution at 37°C for 15 min. The digested tissue was re-suspended in DMEM containing 10% fetal calf serum and mechanically dissociated by gentle aspiration with fire-polished Pasteur pipettes. The dissociated cell suspension was plated at high density (>60,000 cells·cm^{-2}) on pre-coated poly-L-lysine (1 mg·ml^{-1}) 12-mm glass coverslips. Cultures were maintained 2 DIV in an incubator with (1:1) DMEM:Ham's F12 nutrient mixture-astrocyte conditioned media.

Astroglial cultures were prepared as above. Briefly, mesencephalic tissue was chemically and mechanically digested and re-suspended in DMEM-10% fetal calf serum. After that, cells were plated in 25-cm^2 sterile flasks at high density and maintained in an incubator until a confluent monolayer was established around 11–15 DIV. The DMEM: Ham's F12 media conditioned for 48 hr by the astrocyte cultures was used to feed hypothalamic neurons. All cultures were maintained under phenol red-free conditions to avoid "oestrogen-like effects" (Berthois, Katzenellenbogen, & Katzenellenbogen, 1986). In some experiments, male and female hypothalamic cultures were treated with 10-nM **testosterone** immediately after plating until completing 2 DIV (see figure legends for details). Testosterone was diluted in culture media to an appropriate concentration with 10-mM ethanol stock. The final concentration of ethanol in the culture media never exceeded 0.001%.

2.2 | Experimental protocol

All the studies were designed to generate groups of equal size, using randomization and blinded analysis.

2.3 | Calcium imaging

Culture medium was replaced with artificial cerebrospinal fluid (aCSF) containing (in mM) 150 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, 10 sucrose, and pH 7.3. Then, neurons were loaded with 3 μM of the Ca^{2+} indicator Cal-520 (AAT Bioquest Inc., California, USA) for 30 min at 37°C. After that, neurons were washed twice with aCSF and incubated with culture medium for 30 min prior to imaging. Inhibitors (**bicuculline** or **nifedipine**) were added during this time in some experiments.

Coverslips containing cells were then mounted in a recording chamber with 500-μl aCSF for live cell imaging using spinning disc microscopy (Olympus optical, Tokyo, Japan) with epifluorescence illumination (150 W Xenon lamp), a microprocessor, a Hamamatsu CCD video camera, and image intensifier at standard parameters (λexcitation = 492 nm, λemission = 514 nm; imaging frequency: 0.5 Hz; exposure time: 50 ms); 20–30 neurons of each sex were morphologically identified using a 63X objective. After 1 min of recording resting Ca^{+2} (baseline), 500 μl of aCSF containing either **GABA** (10 μM), **muscimol** (10 μM), or GABA + inhibitors (50-μM bicuculline or 20-μM nifedipine) at a 2× concentration was manually added and signals recorded for 4 min. In some experiments, after 3 min of GABA stimulation, the aCSF was removed, and GABA + 5-μM **propofol** was added, and Ca^{2+} signals were obtained over a period of 3 min. During the last minute of all recordings and after aCSF removal, cultures were stimulated with 90-mM KCl. Only neurons that responded to KCl stimulation were included for analysis.

After background subtraction, the fluorescence was quantified with the Fiji/ImageJ Time Series Analyzer plug-in (NIH, Bethesda, MD, USA). Fluorescence intensities (F) were normalised by the average baseline (F_0). The ratio F/F_0 represents the cytoplasmic Ca^{2+} signal as a function of time. We documented the number of neurons with response (F/F_0 > 20% of baseline) and without response (F/F_0 < 20% of baseline); peak (maximum fluorescence intensity after drug application); rise time (time [seconds] to reach the peak); and decay time (time [seconds] needed to return to F/F_0 = 20%).

2.4 | Electrophysiology

Cells were placed in a 2-ml chamber and continuously perfused at a rate of 2 ml·min^{-1} with aCSF. The tip resistance of recording pipettes (borosilicate glass, WPI) was 5–7 MΩ after being filled with internal solution containing (in mM) 120 KCl, 4 $MgCl_2$, 10 HEPES, 10 BAPTA, 2 Mg-ATP, 0.5 Na_2-GTP, and pH 7.3 with KOH. For perforated patch-clamp experiments, the same solution also containing gramicidin diluted to a final concentration of 100 μg·ml^{-1} was used. Gramicidin perforation maintains $[Cl^-]_i$ at physiological levels (Ebihara, Shirato, Harata, & Akaike, 1995).

Electrical measurements were carried out at room temperature with an Axopatch-200A amplifier (Axon Instruments, Foster City, CA, USA). Data were sampled at 10 kHz and pass filtered at 2 kHz, digitalised with an A/D Digidata 1000 using pClamp software (pClamp, RRID:SCR_011323, Molecular Devices, Union City, CA, USA). Pipettes were visualised under a microscope (Olympus optical, Tokyo, Japan) and positioned over the cells by micromanipulators. Whole-cell voltage clamp recordings were performed after formation of a GΩ resistance seal and break-in, while perforated patch-clamp recordings were started at least 20 min after cell-attached formation and transient capacitive peak apparition. Pipette and whole-cell capacitance and series resistance were compensated using amplifier circuitry. Cells were clamped at −50 mV, and only those that presented inward

currents in response to a voltage ramp (−80 to +30 mV) and less than 100 pA of leak current were included for analysis.

Drugs were diluted in aCSF to an appropriate concentration with 10-mM DMSO or distilled water stocks. Drug delivery was performed using a gravity-driven system connected to a capillary HPLC that was positioned 50 μm from the recorded neuron. Neuronal responses to 1-s exposure to the drugs were assessed every minute. Perforated patch-clamp recordings were used to obtain E_{GABA} and $[Cl^-]_i$. Current amplitudes elicited by 10-μM GABA were recorded at different voltages (−60, −50, −40, −30, and −20 mV) to calculate E_{GABA} and $[Cl^-]_i$ in both sexes by means of the Nernst equation. E_{GABA} values were obtained by fitting a second-order exponential curve to the I/V relationship for each neuron. Whole-cell patch-clamp recordings were used to measure neuronal evoked currents by GABA (1 to 500 μM), muscimol (10 μM), and co-application of GABA (10 μM) and different allosteric modulators, such as propofol (5 μM), **alfaxalone** (50 μM), **furosemide** (500 μM), $ZnCl_2$ (1 μM), $LaCl_3$ (100 μM), **THIP** (1 μM,), **Ro 15-4513** (0.3 μM), **diazepam** (1 μM), and **ethanol** (100 mM).

The rationale behind the modulators used in this study was based on their ability to identify $GABA_A$ receptor subtypes by characterising their responses to each drug. Diazepam potentiation depends on γ2 and either α1, α2, α3, or α5 subunits in $GABA_A$ receptors. Furosemide is a diuretic and a strong inhibitor of $GABA_A$ receptors formed by α4β2/3γ2 and α6β2/3γ2, whereas α1/2/3/5β2/3γ2 conformations are practically insensitive. THIP works as a partial agonist of α4βγ2 receptors; a total agonist effect is observed in α4βδ receptors. Ro 15-4513, another synthetic agonist, enhances currents in α4βγ2/α6βγ2 and α6βδ/α4βδ receptors. Similarly, La^{+3} blocks only the $GABA_A$ receptors made up of α6β3γ2L/α6β3δ and α4β3γ2/α4β3δ subunits. On the other hand, the efficacy of the synthetic neurosteroid alfaxalone is higher in δ- $GABA_A$ receptors than in γ2- $GABA_A$ receptors. Ethanol produces a mild potentiation in γ2- $GABA_A$ receptors and a high potentiation in δ- $GABA_A$ receptors . Moreover, ethanol has no effect on $GABA_A$ receptors lacking δ or γ subunits. Zn^{2+} is a non-competitive antagonist for receptors expressing αβε but lacking γ or δ subunits. Propofol potentiates the majority of $GABA_A$ receptor subtypes by a β subunit-dependent mechanism. Particularly, ε-$GABA_A$ receptors show some resistance to propofol with effects greater in α3βθε than in α3βε conformations (Johnston, 1996; Korpi et al., 2002; Olsen & Sieghart, 2008).

Current density in $GABA_A$ receptors was determined dividing the current amplitude of a saturating GABA dose (500 μM) by the capacitance of the same neuron. EC_{50} and the Hill coefficient were obtained by fitting the Hill equation to dose–response current amplitudes for each neuron using ORIGIN® software (Northampton, Massachusetts, USA). All measurements were stored in a PC, and offline analyses were performed with Clampfit (Molecular Devices, Union City, CA, USA).

2.5 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology. All treatments were assigned randomly. The data are presented as mean ± SEM or percentage change over control. The Student's *t* test was used to compare the response of male and female neurons to individual treatments. The χ^2 test was used to compare the proportion of cells with or without response to a specific drug in each sex. Power analysis was set at $P < .05$ value. The statistical analysis for each experiment was performed with data obtained from at least three independent cultures and was undertaken only when each group size was at least $n = 5$ neurons. All statistical analysis was performed with STATISTICA software (STATISTICA, RRID: SCR_014213, StatSoft Inc., Tulsa, OK, USA).

2.6 | Materials

The following compounds were obtained from Tocris Bioscience (Bristol, UK): bicuculline, Ro 15-4513 and THIP. Diazepam was supplied by Laboratorios Duncan (Buenos Aires, Argentina) and ethanol by Merck (Darmstadt, Germany). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.7 | Nomenclature of target and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Kelly et al., 2019; Alexander, Mathie et al., 2019).

3 | RESULTS

3.1 | GABA elevates intracellular Ca^{2+} levels differentially in male and female neurons

To evaluate sex-associated responses of immature neurons after GABA stimulation, hypothalamic neurons were cultured for 2 DIV, a period in which most neurons display a symmetric arrangement of short neurites (minor processes) and one single axon (two to three times longer than minor processes; Cambiasso et al., 1995, 2000). Accordingly, we measured GABA-mediated depolarisation by recording intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), using the fluorescent probe Cal-520 in live neurons (Figure 1). Acute stimulation with GABA (10 μM) produced depolarising responses that were more heterogeneous in males than in females, suggesting sex-associated variability (Figure 1a). Moreover, GABA increased $[Ca^{2+}]$i in most of the male neurons (95%), in contrast to 76% detected in females (Figure 1b). Only 20% of females did not return to basal levels (Figure 1c), suggesting longer lasting depolarising responses in males. Although peaks in $[Ca^{2+}]$i did not differ between sexes (Figure 1d), almost 50% of male neurons did

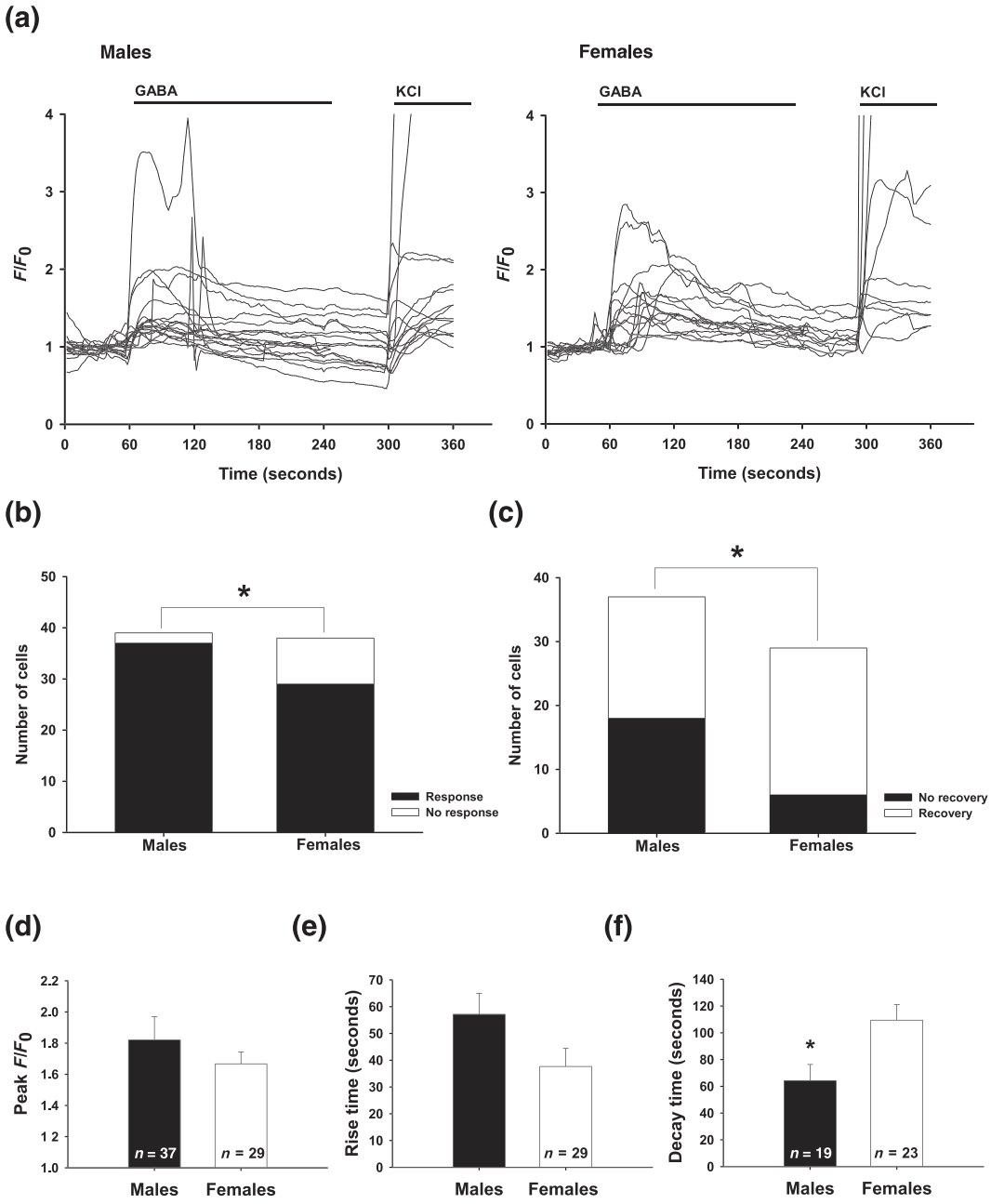

FIGURE 1 GABA stimulation induced intracellular calcium increases. (a) Cal-520 fluorescence (F/F_0) measured in single cells after 10-μM GABA and 90-mM KCl stimulation in both male and female hypothalamic neurons at 2 DIV. Each trace represents a time-dependent fluorescence signal measured in a single neuron soma. Bars represent drug exposure time. (b) Number of neurons responding to GABA in male (37/39) and female (29/38) cultures. $\chi^2 = 5.41$; $^*P < .05$, significantly different as indicated. (c) Number of neurons incapable of recovering basal Ca^{2+} levels in male (18/37) and female (6/29) cultures. $\chi^2 = 5.49$; $^*P < .05$, significantly different as indicated. (d) Maximum fluorescence intensity (F/F_0, peak), (e) rise time (seconds), and (f) decay time (seconds) of the calcium signal induced by 10-μM GABA stimulation in male and female hypothalamic neurons. Values represent the means ± SEM. $^*P < .05$, significantly different from female values; Student's *t* test

not recover their resting $[Ca^{2+}]$i after stimulation. No sex differences were detected in $[Ca^{2+}]$i rise time (Figure 1e). Finally, male neurons that returned to basal $[Ca^{2+}]$i after GABA stimulation exhibited shorter decay times than females (Figure 1f), suggesting differences in neuronal populations, according to $GABA_A$ receptor responses. Together, these results suggest differences in the depolarising effects of GABA between male and female neurons, before the organisational effect of gonadal steroids in utero.

3.2 | $GABA_A$ receptor-induced $[Ca^{2+}]_i$ increases are mediated by L-type VDCC

To explore whether $[Ca^{2+}]_i$ increases were mediated by $GABA_A$ receptors, we used muscimol, a potent selective agonist for these receptors, to stimulate hypothalamic neurons. Neurons were treated acutely with 10-μM muscimol, which raised $[Ca^{2+}]_i$ similarly in males and females (Figure 2a,b). Moreover, co-application of 10-μM GABA and 50-μM

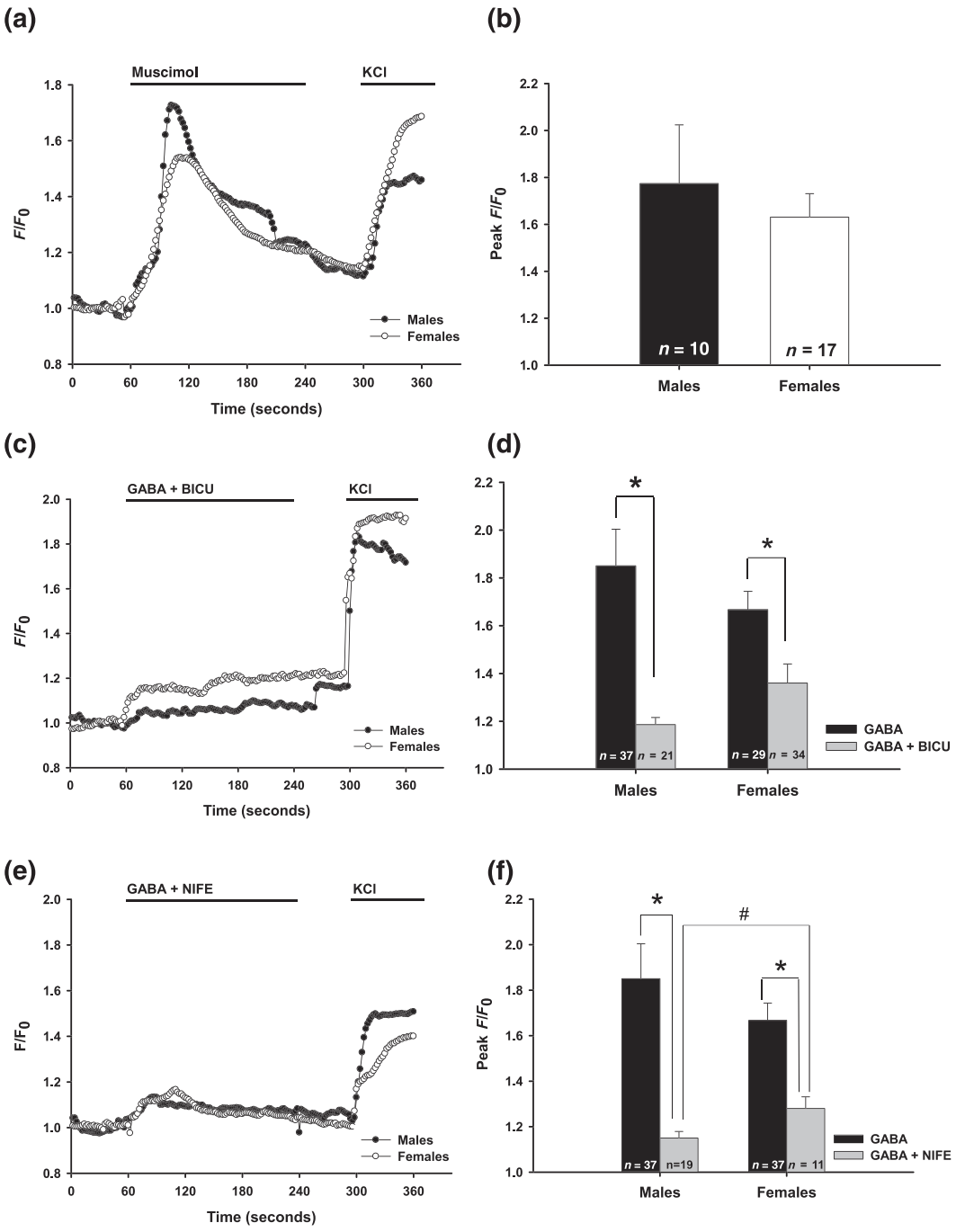

FIGURE 2 $GABA_A$ receptor-mediated intracellular calcium increase depends on L-type VDCC. Mean Cal-520 fluorescence (F/F_0, average of neuronal population) after stimulation with (a) 10-μM muscimol, a potent agonist of $GABA_A$ receptors, (c) 10-μM GABA + 50-μM bicuculline (BICU), a competitive antagonist of $GABA_A$ receptors, and (e) 10-μM GABA + 20-μM nifedipine (NIFE), a specific L-type VDCC blocker, in cultured male and female hypothalamic neurons. Bars represent drug exposure time. (b) Maximum amplitudes (peak) of Ca^{2+} signals after 10-μM muscimol stimulation in male and female neurons. Values represent the mean ± SEM. (d) Maximum amplitudes (peak) of Ca^{2+} signals after 10-μM GABA stimulation and 10-μM GABA + 50 μM bicuculline in male and female neurons. Values represent the mean ± SEM. $^*P < .05$, significantly different from GABA alone; Student's *t* test. (f) Maximum amplitude (peak) of Ca^{2+} signals after 10-μM GABA stimulation and 10-μM GABA + 20-μM nifedipine of male and female neurons. Values represent the means ± SEM. $^{\#}P < .05$, significantly different from females; $^*P < .05$ significantly different from GABA alone; Student's *t* test

bicuculline, a competitive $GABA_A$ receptor antagonist, blunted $[Ca^{2+}]_i$ increases (Figure 2c,d) in both males and females, suggesting that GABA enhances $[Ca^{2+}]_i$ by $GABA_A$ receptors. Then, we studied whether GABA-dependent Ca^{2+} influx could be mediated by VDCC. For this, neurons were pretreated with 20-μM nifedipine, a specific L-type VDCC blocker, and then stimulated with a solution containing both GABA and nifedipine. This inhibited Ca^{2+} influx (Figure 2e) in both male and female neurons, although with a stronger effect in males (Figure 2f).

3.3 | E_{GABA} and $[Cl^-]_i$ do not differ between male and female hypothalamic immature neurons

Considering that the depolarising effects of GABA are mainly attributed to high $[Cl^-]_i$, which produces a reversal potential of $GABA_A$ receptor ion currents (E_{GABA}) that is positive to RMP, we performed perforated patch-clamp configuration experiments to establish the E_{GABA} and $[Cl^-]_i$ in male and female hypothalamic neurons. We registered electrical currents evoked by GABA at several membrane potentials (from −60 to −20 mV, 10-mV steps) in voltage-clamp configuration to obtain an I/V relationship for each neuron. In Figure 3a, we show that, at −60 and −50 mV, GABA stimulation produced an inward current, whereas outward currents were detected at −40, −30, and −20 mV, showing the reversal of $GABA_A$ receptor-mediated responses. E_{GABA} values for male and female neurons were −39.5 ± 3.8 mV and −42.7 ± 2.3 mV respectively. Furthermore, the $[Cl^-]_i$ detected was 35.5 ± 5.5 mM (n = 6) for male and 30.2 ± 2.5 mM (n = 6) for female neurons. Of note, none of these parameters differed between males and females, suggesting that the sex differences observed in depolarising responses by Ca^{2+} imaging cannot be attributed to variations in Cl^- electrochemical gradient force between sexes (Figure 3b).

3.4 | Characterisation of $GABA_A$ receptor-mediated responses and the intrinsic membrane properties of immature hypothalamic neurons

For a comprehensive characterisation of $GABA_A$ receptor-mediated responses in male and female neurons, we measured current amplitude in response to 10-μM GABA in a whole-cell voltage-clamp

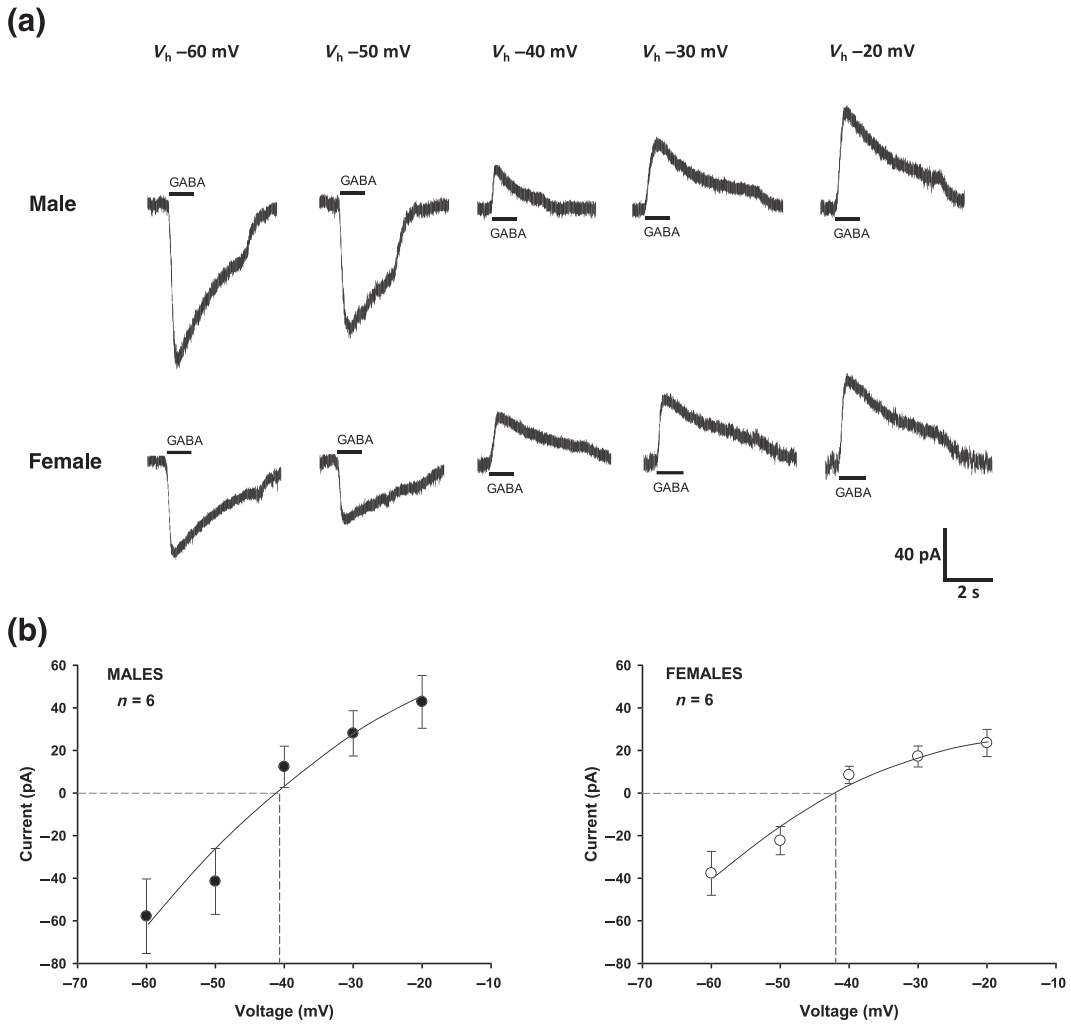

FIGURE 3 Reversal potential of GABA elicited currents. (a) Representative traces of a male and a female hypothalamic neuron after 2 DIV in gramicidin perforated patch-clamp configuration. Graphs shows 10-μM GABA-evoked currents (pA) at different membrane voltages (V_h), showing the reversal of currents from inward to outward. Bars represent ligand exposure time. (b) Current against voltage (I/V) plots of GABA-evoked currents recorded in the gramicidin-perforated patch configuration of male and female neurons. Mean peak current amplitude evoked by GABA (10 μM), registered at potentials varying from −60 to −20 mV by 10-mV steps, was plotted against the membrane potential and a second order exponential curve was fitted to the I/V relationship

(−50 mV) configuration. The inward current detected after GABA stimulation completely disappeared upon ligand removal. Current amplitudes were highly variable among male and female neurons, although our analysis did not reveal any sex difference in their GABAergic current amplitude values (males: 233 ± 30 pA, *n* = 7 vs. females: 155 ± 31 pA, *n* = 9). Moreover, muscimol (10 μM) did not enhance $GABA_A$ receptor currents differently between male and female neurons (300 ± 47 pA, *n* = 7 vs. 218 ± 32 pA, *n* = 9, respectively).

We also analysed several intrinsic membrane properties such as RMP (which ranged from −30 to −57 mV in both sexes), capacitance and membrane resistance, to compare male and female electrical responses; however, no differences were detected between sexes (Table 1). Therefore, neuronal membrane voltage was set at −50 mV to simplify further comparative analysis. We also estimated $GABA_A$ receptor current density, establishing a ratio between the current amplitude evoked by a saturating dose of GABA (500 μM) and the capacitance of the same neuron; this ratio is proportional to $GABA_A$ receptor levels. Average current amplitudes recorded were 607 ± 104 pA and 530 ± 175 pA for males and females respectively. Moreover, mean $GABA_A$ receptor current density values were 55.8 ± 7.6 pA/pF for males (*n* = 10) and 52.5 ± 15.5 pA/pF for females (*n* = 10). Together, these data suggest that male and female neurons share similar levels of active $GABA_A$ receptors at this developmental stage.

3.5 | Pharmacological characterisation of $GABA_A$ receptors in male and female hypothalamic neurons

The physiological properties of $GABA_A$ receptors vary depending on its subunit composition. Therefore, we performed an exhaustive functional characterisation of male and female $GABA_A$ receptors to explore sex-dependent composition.

3.6 | GABA sensitivity

First, we studied GABA sensitivity in male and female hypothalamic neurons at 2 DIV, through a concentration–response analysis; Figure 4a shows that the higher the concentration of GABA, the higher the current registered, revealing more recruitment of $GABA_A$ receptors . A weak response was elicited by the lowest concentration (1 μM) as well as fast desensitisation after 500-μM GABA stimulation. There was high variability in EC_{50} values in both sexes (ranging from 6.1 to 66.7 μM), suggesting intrinsic variability in the GABA sensitivity of immature cultured neurons. Of note, neither EC_{50} nor Hill coefficients (males: 1.45 ± 0.19, *n* = 14 and females: 2.94 ± 1.98, *n* = 11) differed between sexes (Figure 4b).

TABLE 1 Intrinsic membrane properties of male and female hypothalamic neurons after 2 DIV

Parameter	Males	Females
Resting membrane potential (mV)	−40.5 ± 2.31 (11)	−42.4 ± 2.68 (9)
Membrane resistance (MΩ)	257.35 ± 40.8 (9)	238.28 ± 24.68 (7)
Capacitance (pF)	10.41 ± 0.71 (10)	10.56 ± 1.31 (10)

Note. Values represent mean ± SEM; (*n*) number of neurons.

3.7 | Allosteric modulator responses

Next, we compared the $GABA_A$ receptor responses of male and female neurons treated with a battery of allosteric modulators (co-applied with 10-μM GABA) and then analysed their enhancer/inhibitory effect over currents. A modulator was considered effective if it was able to modify currents within a range of ±10% compared to the 10-μM GABA response of the same neuron (Figure 5a). Accordingly, either potentiation or inhibition values were estimated by pooling neurons with similar responses (up or down respectively; Figure 5b).

Almost all neurons were sensitive to 1-μM diazepam and to the synthetic steroid alfaxalone (50 μM). Co-application of 100-μM $LaCl_3$ and 10-μM GABA also induced similar current potentiation in neurons from males and females. In contrast, furosemide (500 μM) inhibited GABA-mediated currents in around 50% of neurons, equally in male and females. Several other modulators assessed in this study did not modify GABA-evoked currents, including 1-μM THIP or 0.3-μM Ro 15-4513. Moreover, a low concentration of Zn^{2+} (1 μM), co-applied with GABA, blunted currents in half of the neurons, to the same extent in males and females. Overall, we detected no differences in potentiation or inhibition effects between sexes after allosteric modulators, except for propofol (see below).

Our results also showed that 100-mM ethanol may boost or block evoked currents by 10-μM GABA in hypothalamic neurons (Figure 6a), with equal enhancement or inhibition in males and females (Figure 6b). However, a considerable number of neurons were insensitive to ethanol treatment. Our statistical analysis revealed no differences between sexes in either the frequency of responding cells or the enhancer/inhibitor effect of ethanol.

Propofol is an anaesthetic that potentiates the action of GABA at most $GABA_A$ receptor subtypes, although some resistance is observed in $GABA_A$ receptors containing ε subunits (Davies, Hanna, Hales, & Kirkness, 1997; Davies, Kirkness, & Hales, 2001). In our model, while 5-μM propofol enhanced GABA currents in most male or female hypothalamic neurons, 30% remained insensitive or were slightly inhibited. Moreover, the potentiation achieved by propofol was greater in female than in male neurons (Figure 5), suggesting differences in $GABA_A$ receptor subunits between sexes. Table S1 summarises our main conclusions regarding the $GABA_A$ receptor subtypes detected in male and female neurons based on this screening.

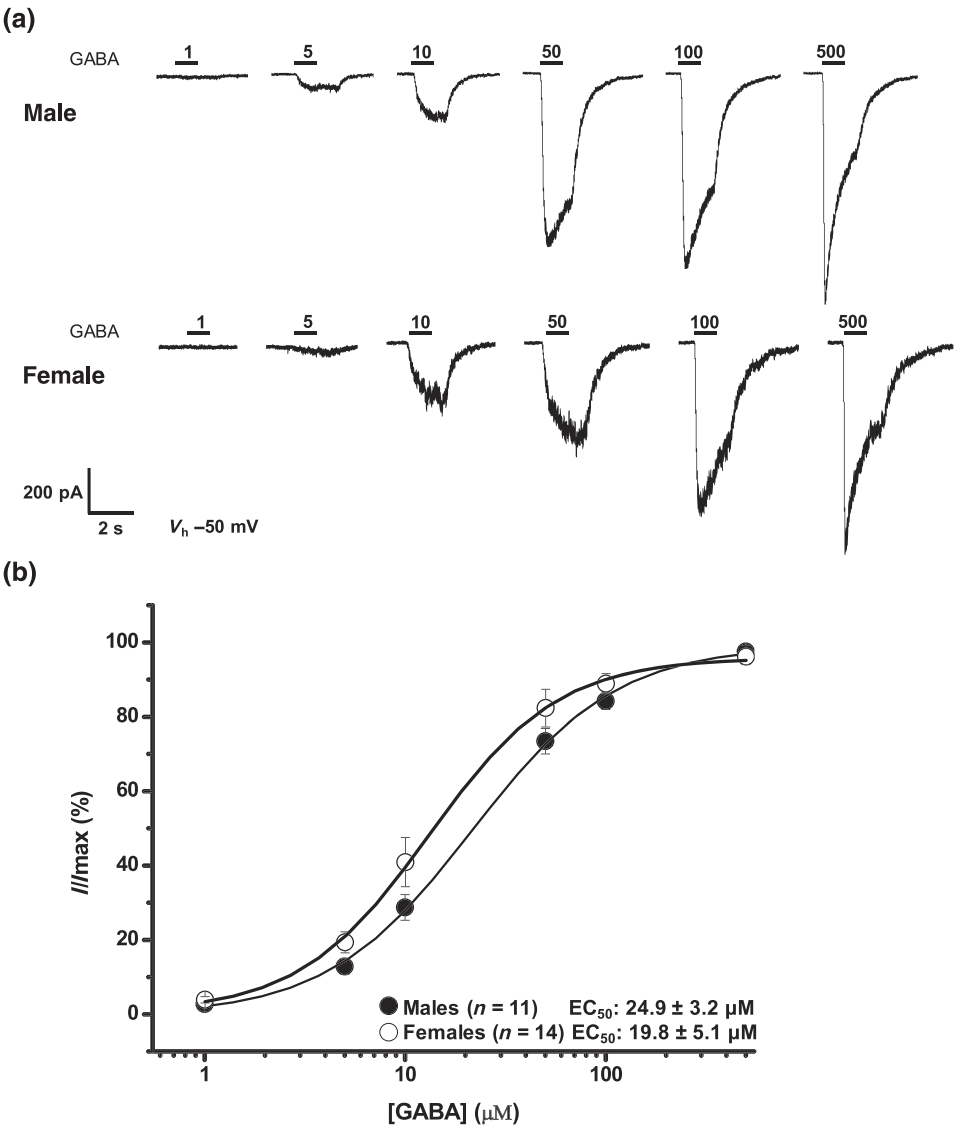

FIGURE 4 Concentration–response relationships induced by GABA on male and female neurons in culture. (a) Representative traces showing dose–response currents after GABA stimulation (1–500 μM) of a male and a female neuron (V_h = holding potential). Bars represent ligand exposure time. (b) Dose–response curve to GABA of male and female hypothalamic neurons. I/I_{max} (%): current percentage with respect to maximum current. EC_{50} values (μM) are means ± SEM

3.8 | Effect of propofol on $GABA_A$ receptor-mediated $[Ca^{2+}]_i$ increases

As propofol (5 μM) showed a selective enhancement in female neurons, we explored whether co-stimulation with GABA (10 μM) would increase Ca^{2+} influx. However, this strategy blunted a $GABA_A$ receptor-mediated $[Ca^{2+}]_i$ increase, a phenomenon observed only in female neurons (Figure 7a,b).

3.9 | Testosterone does not modify sexually dimorphic depolarising $GABA_A$ receptor responses

Our data showed sex differences in GABA-mediated depolarisation in hormone-naïve neurons. Therefore, we wondered whether these differences were maintained when neurons are treated with testosterone, mimicking the in utero surge of testosterone during the critical period. For this, we treated cultures of male and female hypothalamic neurons with 10-nM testosterone immediately after plating, until completing 2 DIV and then evaluated Ca^{2+} influx after GABA stimulation. A global effect was detected, revealed by an increase in the number of neurons effectively depolarised by KCl (90 mM) and GABA (10 μM; Figure 8a). Surprisingly, the sex differences in $GABA_A$ receptor responses detected in hormone-naïve neurons were maintained in testosterone-treated cultures. Moreover, most of the male neurons did not recover their basal $[Ca^{2+}]_i$ after GABA stimulation, a phenomenon occurring in about half of female neurons (Figure 8b). However, those neurons that returned to their basal $[Ca^{2+}]_i$ showed similar decay times (males: 104 ± 18 s, n = 2; females: 104 ± 4 s, n = 28). However, testosterone increased the $[Ca^{2+}]_i$ peak value after GABA depolarisation, more in neurons from males than in females (Figure 8c) and a similar effect was observed in rise time values (Figure 8d). Suprisingly, testosterone potentiated the effects of propofol on hypothalamic neurons. While depolarising effects increased in males, a blockade was detected in female neurons (Figure 8e). This suggests that testosterone not only does not erase differences between sexes, but increases the effect detected in neurons without hormone exposure.

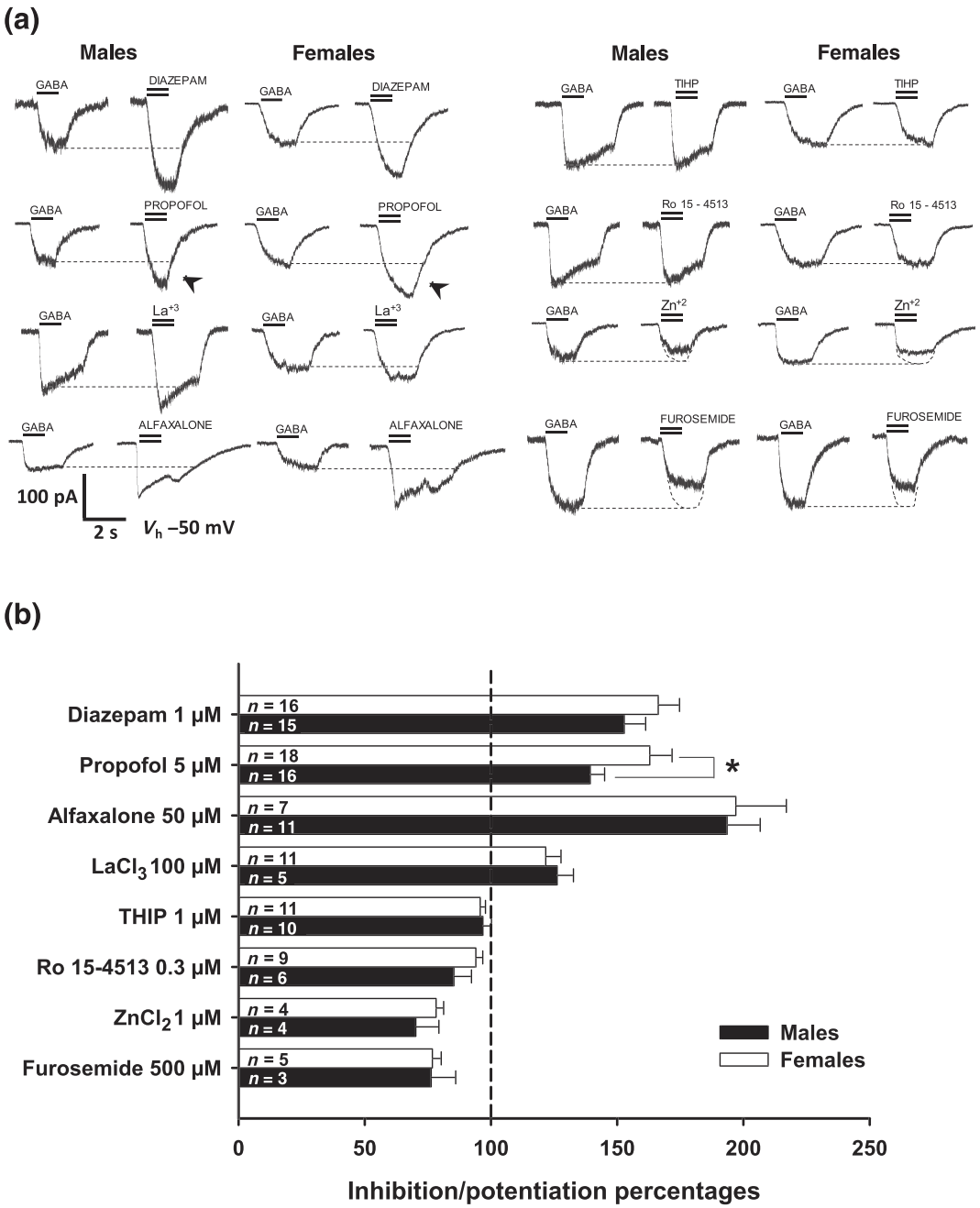

FIGURE 5 Allosteric modulation of $GABA_A$ receptors by pharmacological agents. (a) Representative traces showing electrical currents after GABA stimulation and several allosteric modulators in 2 DIV male and female hypothalamic neurons (V_h = holding potential). Bars represent drug exposure time. Arrow head indicates the stronger effect of propofol modulation in females than in males. (b) Inhibition or potentiation percentages of 10-μM GABA-evoked currents co-applied with 1-μM diazepam (males 15/16, females 16/17), 5-μM propofol (males 16/22, females 18/28), 50-μM alfaxalone (males 11/11, females 7/7), 100-μM $LaCl_3$ (males 5/5, females 11/17), 1-μM THIP (males 0/10, females 0/11), 0.3-μM Ro 15-4513 (males 0/6, females 0/9), 1-μM $ZnCl_2$ (males 4/9, females 4/7), and 500-μM furosemide (males 3/7, females 5/8). Dotted line represents GABA (10 μM) applied alone (number of responsive neurons/total neurons registered). Values represent the means ± SEM. *$P < .05$, significantly different as indicated; Student's *t* test

4 | DISCUSSION

Gonadal hormones are the main factors mediating brain sexual differentiation (McCarthy et al., 2009). However, the genetic background derived from different sex chromosome complement has marked effects on neuronal development, connectivity, and function (Arnold, 2017). In fact, X/Y-linked genes directly affect hypothalamic sexual differentiation at molecular and behavioural levels, regardless of hormonal environment (Büdefeld et al., 2010; Grgurevic et al., 2012; Majdic & Tobet, 2011). Although previous studies have

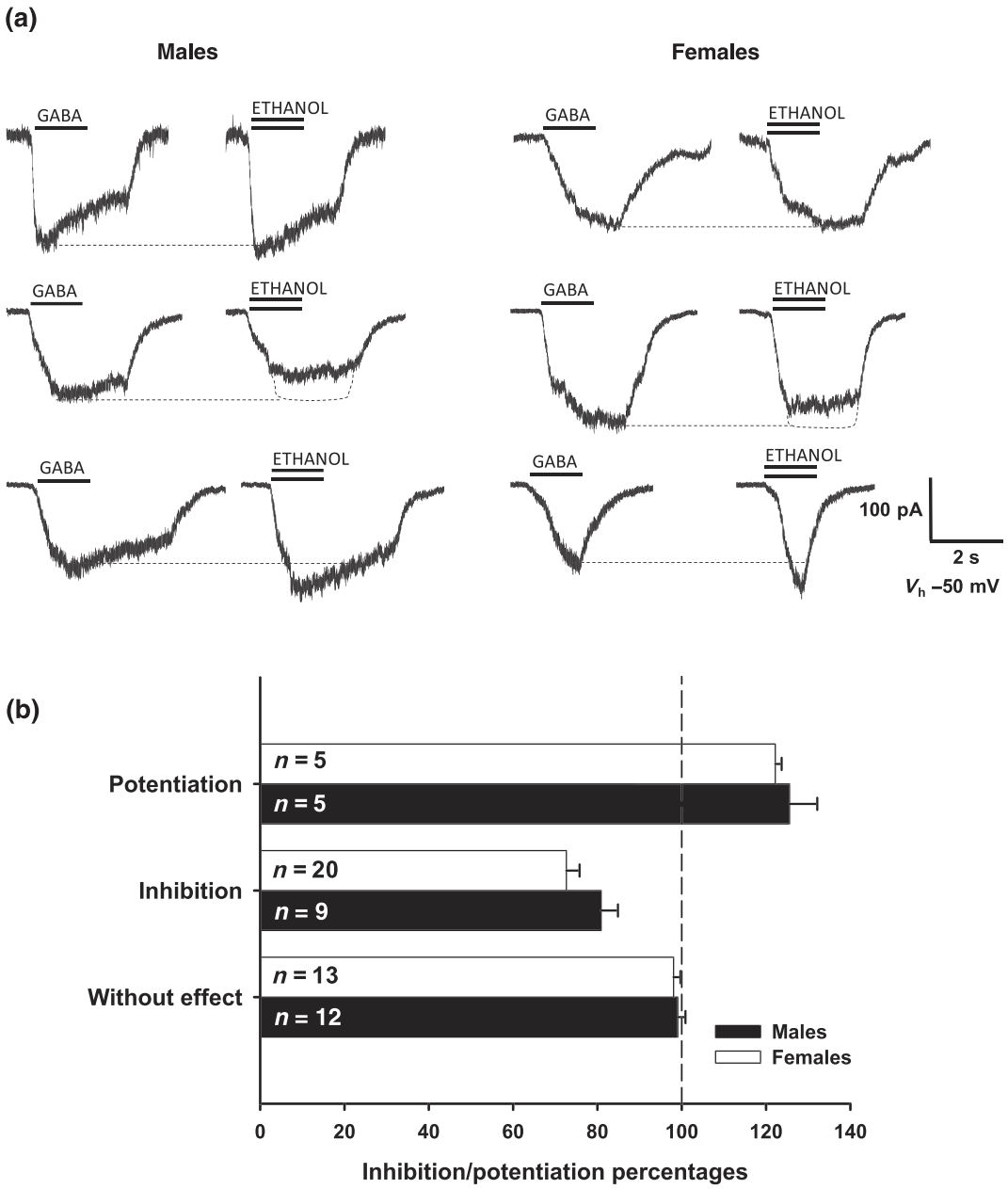

FIGURE 6 Modulation of $GABA_A$ receptor responses by ethanol. (a) Representative traces of GABA-elicited currents (10 μM) and ethanol modulation (100 mM) of male and female hypothalamic neurons after 2 DIV (V_h = holding potential). Bars represent drug exposure time. (b) Inhibition or potentiation percentages of 10-μM GABA-evoked currents co-applied with 100-mM ethanol in male and female hypothalamic neurons. Dotted line represents 10-μM GABA applied alone

reported sex differences in several GABA parameters from both in vitro and in vivo hypothalamic neurons, these differences were linked to gonadal hormone-dependent effects (Auger et al., 2001; Davis et al., 1999; Kellogg et al., 2000; Perrot-Sinal et al., 2007; Smith et al., 1996; Zhou et al., 2005).

In this work, we showed that sex differences in GABA responses of hypothalamic neurons are manifested before the critical period of brain masculinisation, mostly independent of hormonal treatment. Our data suggest that a greater number of male than female neurons were depolarised by GABA (as shown by Ca^{2+} influx), exhibiting depolarising responses lasting longer periods of time (Figure 1). These results are consistent with previous evidence obtained in 9 DIV neurons (Mir et al., 2017), suggesting that sex differences are established early in neuronal development, even before the peak of testosterone levels at E18. We also found that $GABA_A$ receptors mediate Ca^{2+} influx, membrane depolarisation, and L-type VDCC opening (Figure 2), the canonical pathway by which GABA excites immature neurons (Ben-Ari, 2002). Surprisingly, the inhibition of Ca^{2+} influx by nifedipine was stronger in male than in female neurons (Figure 2f), suggesting differences in regulatory mechanisms for L-type VDCC. In this regard,

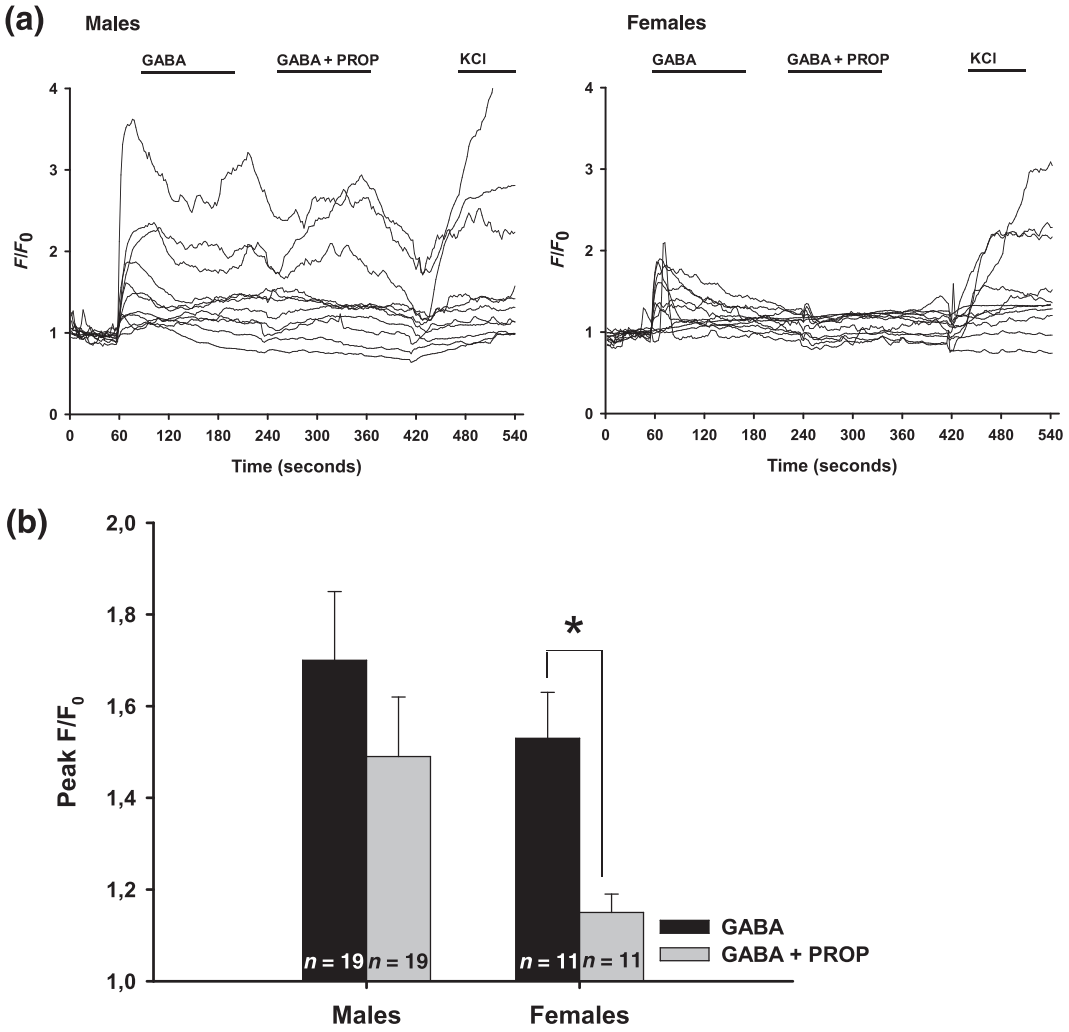

FIGURE 7 Propofol-dependent modulation of $GABA_A$ receptor induces intracellular calcium increases. (a) Cal-520 fluorescence (F/F_0) measured in single cells after 10-μM GABA, 10-μM GABA + 5-μM propofol (PROP), and 90-mM KCl stimulation in both male and female hypothalamic neurons at 2 DIV. Each trace represents a time-dependent fluorescence signal measured in a single neuron soma. Bars represent drug exposure time. (b) Maximum amplitudes (peak) of Ca^{2+} signals after 10-μM GABA and 10-μM GABA + 5-μM propofol treatments in male and female cultured hypothalamic neurons. Values represent the means ± SEM. *P < .05, significantly different as indicated; Student's t test

sex differences in depolarising $GABA_A$ receptor actions cannot be assigned to differences in Cl^- electrochemical force, GABA sensitivity, or $GABA_A$ receptor number between sexes, since both E_{GABA} and $[Cl^-]_i$ (Figure 3), EC_{50} GABA (Figure 4b), and $GABA_A$ receptor current density (an indirect measure of the number of receptors) were similar in both sexes. However, our pharmacological screening supports a hypothesis related to a sex-dependent composition of $GABA_A$ receptor subtypes.

Considering the responses recorded using allosteric modulators, as well as by expression profiles previously published (Laurie et al., 1992; Pape et al., 2009), we conclude that both male and female hypothalamic neurons possess a large variety of functional $GABA_A$ receptors (Figures 5 and 6; Johnston, 1996; Korpi et al., 2002; Olsen & Sieghart, 2008). From our data, we would hypothesise the coexistence of several populations of hypothalamic neurons in culture, an observation also supported by the high variation of GABA-mediated responses (even within each sex). Accordingly, we infer that almost all 2 DIV neurons display α2β2/3γ2, α3β2/3γ2, and α5β2/3γ2 conformations of $GABA_A$ receptors. Nevertheless, a subpopulation (around 50%) could also display functional $GABA_A$ receptor containing α4, α3, θ, and/or ε subunits. It should be noted that we also found that propofol-dependent current potentiation was higher in females than in males (Figure 5) but that propofol acted as a potent blocker of Ca^{2+} influx mediated by GABA in females but not in males (Figure 7), suggesting sex differences in $GABA_A$ receptor subunit composition. In other words, this propofol-dependent effect may reveal differences between sexes in β, θ, and/or ε $GABA_A$ receptor composition (Table S1). Thus, it is important to highlight that administration of testosterone did not erase sex differences, either in $GABA_A$ receptor-dependent depolarisation or in propofol response (Figure 8), suggesting hormone-independent effects. In fact, testosterone treatment increased the sex differences reported in this study.

Activation of $GABA_A$ receptors is the main excitatory signal for embryonic developing circuits, modulating Ca^{2+} -mediated processes

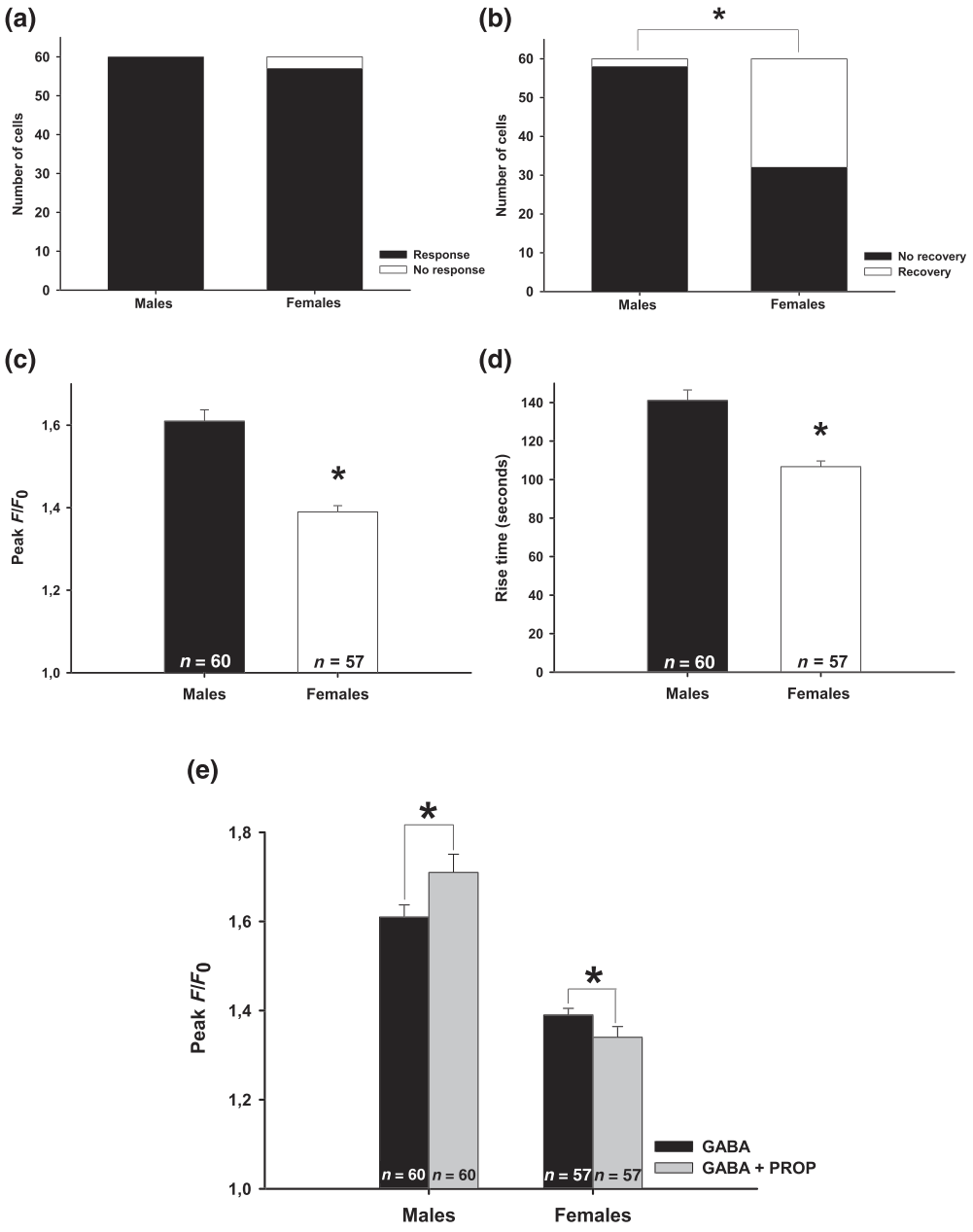

FIGURE 8 Testosterone treatment enhances the sex differences in GABA-evoked intracellular calcium increases. Male and female hypothalamic neurons were treated with 10-nM testosterone after plating and cultured until completing 2 DIV. (a) Number of neurons responding to GABA in male (60/60) and female (57/60) cultures. (b) Number of neurons incapable of recovering basal Ca^{2+} levels in male (58/60) and female (32/60) cultures. $\chi^2 = 30.04$; *$P < .05$, significantly different as indicated. (c) Maximum fluorescence intensity (F/F_0, peak) and (d) rise time (seconds) of fluorescence calcium signal induced by 10-μM GABA stimulation in male and female hypothalamic neurons previously treated with testosterone. Values represent the means ± SEM. *$P < .05$, significantly different from male values; Student's *t* test. (e) Maximum amplitudes (peak) of Ca^{2+} fluorescence signals after 10-μM GABA and 10-μM GABA + 5-μM propofol treatments in male and female cultured neurons previously treated with testosterone. Values represent the means ± SEM. *$P < .05$, significantly different from GABA alone; Student's *t* test

such as neuronal differentiation, neurite outgrowth, and survival (Represa & Ben-Ari, 2005; Sernagor, Chabrol, Bony, & Cancedda, 2010). Earleir work had shown that propofol, through $GABA_A$ receptors and L-type VDCC activation, modified the axonal and dendritic morphology of cortical neurons (Briner et al., 2011; Mintz, Barrett, Smith, Benson, & Harrison, 2013) and also produces cell death of hippocampal neurons (Kahraman, Zup, McCarthy, & Fiskum, 2008). Moreover, male hippocampal neurons were more vulnerable than those of females to $GABA_A$ receptor-dependent excitotoxicity, apparently due to failures in switching-off Ca^{2+} transients elicited by $GABA_A$ receptor over-activation. This effect has been mainly attributed to a hormone-dependent sex difference of $GABA_A$ receptor subunits (Nuñez & McCarthy, 2008). Nevertheless, differences detected in our study are independent of testosterone treatment and most probably dependent on sex chromosome complement.

Considering our results blocking L-type VDCC with nifedipine in male and female neurons, we do not discount sex differences in the composition and expression of these channels (Figure 2f). In fact, propofol inhibits L-type VDCC by a voltage-dependent inactivation mechanism (Fassl, High, Stephenson, Yarotskyy, & Elmslie, 2011; Martella et al., 2005), which could explain the selective inhibition by propofol in females (Figure 7). Several reports support the notion that sex chromosomes encode many transcription factors regulating both autosomal and sexual genes, leading to imbalances in gene expression between XX (female) and XY (male) cells (Carrel & Willard, 2005; Lee & Bartolomei, 2013; Wijchers & Festenstein, 2011). In fact, the cluster of genes encoding α3/θ/ε $GABA_A$ receptor subunits (Simon

et al., 2004) and the $Ca_v1.4$, L-type VDCC-subunit gene (Catterall, Perez-Reyes, Snutch, & Striessnig, 2005) are located on the X chromosome, and their expression could be different in XX and XY hypothalamic neurons.

The hypothalamus is one of the most sexually dimorphic regions in the brain, controlling important sexually dimorphic behaviours (Flanagan-Cato, 2011; Griffin & Flanagan-Cato, 2009; Yang et al., 2013). Hypothalamic sex differences are largely connected to early events in development such as proliferation and apoptosis, lineage commitment, neuronal migration, and connectivity. Accordingly, GABAergic signalling, the main excitatory input at embryonic developmental stages (Ben-Ari, 2002), is critical to sustain developmental and physiological aspects of developing neurons before the establishment of synapses. Several trophic and paracrine roles have been described for GABA, ranging from cell proliferation control, migration, neurite outgrowth, and synapse formation (Cancedda et al., 2007; Chudotvorova et al., 2005; Represa & Ben-Ari, 2005; Reynolds et al., 2008; Sernagor et al., 2010). Therefore, the sexually dimorphic depolarising effects of GABA reported in this work could differentially influence the morphology, physiology, and connectivity of male and female hypothalamic neurons, even before exposure to gonadal hormones. Moreover, our pharmacological screening reinforces the importance of considering sex as a key variable for pharmacological studies. Of note, our results suggest sex differences in GABAergic signalling after treatment with propofol, which is regularly used as an anaesthetic. Such an observation has clinical relevance for men's and women's health (Briner et al., 2011; Kahraman et al., 2008; Mintz et al., 2013).

In summary, our work shows that male and female hypothalamic neurons differ in their GABAergic physiology, independent of gonadal hormones. Hormone administration did not erase differences, suggesting that the sexual genetic backgrounds of sexes are the most probable basis for these findings. To our knowledge, this is the first study reporting GABAergic differences between male and female neurons before brain sexual differentiation, and the consequent importance of considering this issue in the biology and physiology of hypothalamic neurons.

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AUTHOR CONTRIBUTIONS

F.R.M., L.G.A., and M.J.C. conceived and designed the research. F.R.M. performed all electrophysiological experiments and analysed the data. F.R.M., C.W., and L.E.C.Z. performed calcium imaging experiments and analysed the data. F.R.M., C.W., L.E.C.Z., L.G.A., and M.J.C. interpreted the results of experiments. F.R.M. elaborated the figures and wrote the manuscript. C.W., L.E.C.Z., L.G.A., and M.J.C. edited and revised the manuscript. M.J.C. drafted the manuscript. This study was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 2013–2015), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2015 No. 1333), Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT-UNC, 2018–2021) to M.J.C., from the Secretaría de Ciencia y Tecnología de la Universidad Nacional de La Rioja (00-10460/2015) to F.R.M., and from Fondecyt—1180753—to L.G.A. C.W. and L.E.C.Z. are postdoctoral and doctoral fellows of CONICET respectively.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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