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MOLECULAR AND SYNAPTIC MECHANISMS

Sex differences in depolarizing actions of $GABA_A$ receptor activation in rat embryonic hypothalamic neurons

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Abstract

$GABA_A$ receptor activation exerts trophic actions in immature neurons through depolarization of resting membrane potential. The switch to its classical hyperpolarizing role is developmentally regulated. Previous results suggest that a hormonally biased sex difference exists at the onset of the switch in hypothalamic neurons. The aim of this work was to evaluate sex differences in $GABA_A$ receptor function of hypothalamic neurons before brain masculinization by gonadal hormones. Hypothalamic cells were obtained from embryonic day 16 male and female rat foetuses, 2 days before the peak of testosterone production by the foetal testis, and grown *in vitro* for 9 days. Whole-cell and perforated patch-clamp recordings were carried out in order to measure several electrophysiological parameters. Our results show that there are more male than female neurons responding with depolarization to muscimol. Additionally, among cells with depolarizing responses, males have higher and longer lasting responses than females. These results highlight the relevance of differences in neural cell sex irrespective of exposure to sex hormones.

Introduction

γ-Aminobutyric acid (GABA) acts trough $GABA_A$ receptors (GABAAR) as the main inhibitory neurotransmitter in adult central nervous system (CNS). However, during early development GABA exerts excitatory actions (Ben-Ari, 2002), being able to fire action potentials (Gao & van den Pol, 2001) and to cause the opening of voltage-dependent calcium channels with the consequent increase in intracellular calcium levels (Obrietan & van den Pol, 1995). Excitatory actions of GABA in immature neurons are important regulators of normal CNS development modulating progenitor proliferation, neuronal migration and differentiation (Represa & Ben-Ari, 2005; Wang & Kriegstein, 2009; Sernagor *et al.*, 2010). The developmental switch of GABA actions takes place around postnatal day 6 (PN6) in the rat hypothalamus (Obrietan & van den Pol, 1995).

Classical hypothesis of brain sexual differentiation establish that male typical organization of brain circuits is a consequence of organizing actions of androgens secreted by foetal testes during the 'critical period' of brain development that in rats extends from embryonic day 18 (E18) to PN10 (Arnold & Gorski, 1984). On the contrary, female brain organization results in absence of high levels of gonadal secretions. Hormonally mediated brain sexual differentiation occurs during the same temporal window in which GABA actions switch from depolarizing to hyperpolarizing and seems to be decisive for the outcome of the switch. A great body of evidence suggests a close relationship between GABA signalling and hormonally mediated sexual differentiation of hypothalamic neurons (McCarthy *et al.*, 2002).

During the last twenty years evidence has accumulated showing that there are sex differences independent of hormonal actions (Arnold, 2004; De Vries, 2004). Particularly our laboratory has contributed with evidence in favour of the existence of sex differences independent of hormones in hypothalamic neurons. Male derived cultures respond different from female cultures to hormonal and glial environment (Cambiasso *et al.*, 1995, 2000). Moreover, sex chromosome complement affects differentially male and female neuronal differentiation and transcription factors expression (Scerbo *et al.*, 2014). Here, we demonstrate that male derived cultures have more neurons with depolarizing responses to muscimol than female cultures. We also show that among cells with depolarizing responses, males have larger and longer lasting responses than females. This sex differences occur before the critical period of brain development and might be due to the different expression of X and Y genes that underlie constitutive genetic differences in male and female neuronal cultures.

Materials and methods

Care and use of laboratory rodents was in accordance with National Institutes of Health guidelines and were approved by Institutional

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Animal Care and Use Committee of the Instituto Ferreyra (CICUAL-IMMF, Córdoba, Argentina).

Cell cultures

Foetal donors were obtained from pregnant Wistar rats (n = 10) at E16 for neuronal and glial cultures. The day of vaginal plug was defined as E0. Pregnant rats were killed by cervical dislocation under CO_2 anaesthesia, and the foetuses were dissected from the uterus. Foetuses were separated by sex trough the identification of the spermatic artery on the developing testes. Under a dissecting microscope, the ventromedial hypothalamic and mesencephalic regions were dissected out and stripped of the meninges for neuronal and glial cultures, respectively. Primary neuronal and glial cultures were prepared and maintained as described elsewhere (Cambiasso *et al.*, 2000). All cultures were raised under phenol red-free conditions to avoid 'oestrogen-like effects' (Berthois *et al.*, 1986). For neuronal cultures, the dissociated cell suspension was plated at high density (60 000 cells/cm^2) on 12 mm glass coverslips (Assistant, Germany) pre-coated with 1 mg/mL poly-D-lysine. Glial cultures were prepared in sterile flasks and maintained in DMEM medium supplemented with 10% Foetal Bovine Serum until a monolayer was present. The basal medium (BM) was (1 : 1) DMEM : Ham's F12 Nutrient Mixture, supplemented with 50 μg/mL gentamycin and conditioned by the astroglial cultures for 48 h. Neurons were maintained for 9 days (9 DIV) in astroglial-conditioned BM in an incubator with 5% CO_2 humidified atmosphere at 37 °C before electrophysiological experiments were conducted.

Electrophysiology

Dishes were placed in a 2 mL chamber fixed to a microscope (Olympus Optical, Tokyo, Japan). Cells were continuously perfused at a rate of 2 mL/min with a bath solution containing (in mM): 150 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, 10 sucrose, pH 7.3. 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, pH 7.3. For perforated patch-clamp experiments, gramicidin stock solution was prepared by dissolving in DMSO (50 mg/mL). The pipette tip was initially filled with gramicidin-free solution (in mM: 145 $KMeSO_4$, 1 $MgCl_2$, 10 HEPES, 11 EGTA, 4 Mg-ATP, 0.5 Na_2-GTP, pH 7.3) by a brief immersion. The remainder of the pipette was then backfilled with the same solution also containing gramicidin diluted to a final concentration of 100 μg/mL. Gramicidin perforation maintains the intracellular Cl^- concentration ($[Cl^-]_i$) at physiological levels (Ebihara *et al.*, 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, digitalized with an A/D Digidata 1000 using pClamp software (Molecular Devices, Union City, CA, USA). Pipettes were positioned by micromanipulators and after formation of a GΩ resistance seal and break-in, whole-cell signals were recorded. At least 20 min after cell-attached formation and transient capacitive peaks apparition, gramicidin-perforated patch recordings were started. 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

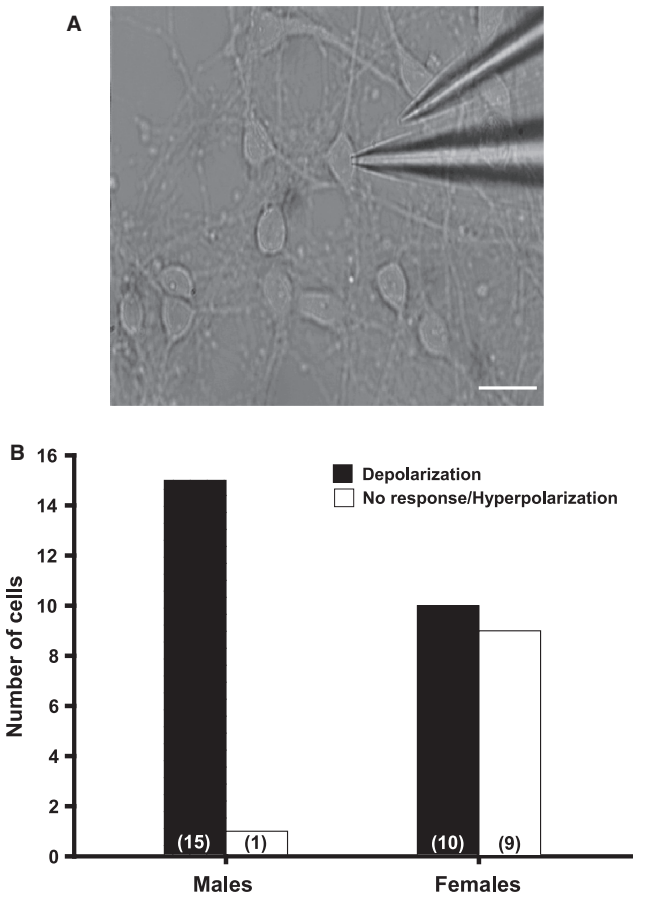

FIG. 1. (A) Male 9 days *in vitro* hypothalamic culture in the recording chamber, register and ejection pipettes are shown. Bar represents 20 μm. (B) Number of cells with depolarization (black) and no response/hyperpolarization (white) responses to muscimol (10 μM) application of male and female neurons. χ^2=7.19; $p < 0.01$. (n) Number of cells.

100 pA of leak current were included for analysis. Perforated patch-clamp recordings were obtained in current-clamp amplifier configuration in order to obtain proportion of cells with depolarizing/hyperpolarizing responses, voltage changes at different holding potentials and E_{Cl^-}. Voltage-clamp configuration was used in whole-cell patch-clamp recordings in order to obtain dose–response curves to GABA and GABAAR current densities ($\delta_{I\text{-}GABAAR}$). Muscimol and GABA were diluted in bath solution in an appropriate concentration from 10 mM distilled water stocks. An ejection pipette of borosilicate ~ 2 μm tip diameter, filled with 10 μM muscimol and connected to a pneumatic pump (Medical Systems Corp., Great Neck, NY, USA) was used for drug delivery. The ejection pipette was positioned 20 μm away from the recorded neuron and peak voltage responses (V_Δ) to 10 pisg, 600 ms duration pulses were recorded at different holding potentials (V_h) (−60, −50, −40, −30 and −20 mV) in current-clamp amplifier configuration. The range of holding potentials chosen was sufficient to cause a reversal in the polarity of the responses. Peak voltage responses were plotted as a function of holding potential (V_Δ/V_h) and the chloride reversal potential (E_{Cl^-}), the holding potential at which the response peak amplitude was 0, determined by interpolation from the equation of a second order exponential curve through the data points (Tunstall *et al.*, 2002). Current clamp was adopted because in perforated patch clamp we

TABLE 1. Instrinsic membrane properties of 9 days *in vitro* male and female hypothalamic neurons

	Males	Females
Resting membrane potential (mV)	−36.13 ± 2.02 (15)	−32.10 ± 2.00 (10)
Membrane resistance (MΩ)	363 ± 80.49 (6)	496 ± 111.24 (5)
Capacitance (pF)	11.70 ± 1.42 (7)	11.29 ± 0.57 (9)

Values represents mean ± SEM; (*n*) number of cells.

obtained a larger signal to noise ratio. $[Cl^-]_i$ was determined by means of the Nernst equation. A gravity-driven system connected to a capillary HPLC was used to obtain the GABA dose–response curve in voltage-clamp experiments. This configuration allowed us to rapidly change the GABA solution (1–500 μM). The capillary was positioned 50 μm away from the recorded neuron and the neuronal response to 1 s exposure to the drug solutions was assessed every minute at −50 mV. Male and female EC50 and Hill coefficient were obtained by fitting the Hill equation to dose–response current amplitudes for each neuron using ORIGIN® software. $\delta_{I\text{-}GABA}$ was determined dividing the current amplitude evoked by a saturating dose of GABA (500 μM) by the capacitance of the same neuron. All reagents were from SIGMA-ALDRICH® unless stated. All measurements were stored in a PC and off-line analyses were performed with Clampfit (Molecular Devices, Union City, CA, USA).

Statistical analysis

ANOVA, followed by *post hoc* LSD test, was used to detect significant differences. Student's *t*-test was used to compare the response of male and female neurons to individual treatments. Chi^2 test (χ^2) was used to compare the proportion of cells with depolarizing or no response/hyperpolarizing responses in each sex. All statistical analyses were performed with STATISTICA software (StatSoft Inc., Tulsa, OK, USA).

Results

Intrinsic membrane properties of male and female hypothalamic neurons

After 9 DIV (Fig. 1A), most neurons present an ovoid soma with one to several neuritic processes which can be classified as dendrites or axons by morphological and immunocytochemical criteria (Díaz *et al.*, 1992). Electrophysiological data are shown in Table 1. No sex differences were found in resting membrane potential (RMP), capacitance (C) and membrane resistance (R) between male and female hypothalamic neurons.

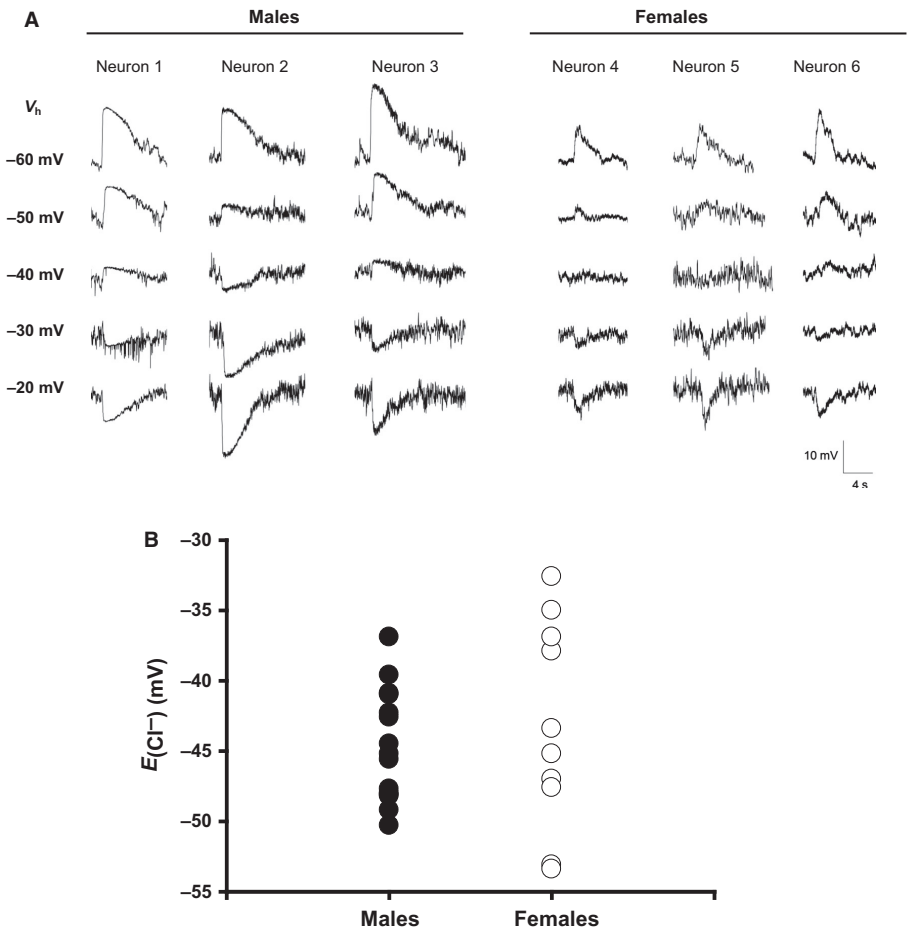

FIG. 2. (A) Representative membrane potential changes (mV) in response to 10 μM muscimol application at different holding potentials (V_h) in male and female 9 days *in vitro* hypothalamic neurons in current-clamp amplifier configuration. (B) Chloride reversal potentials (E_{Cl^-}) of male (n = 15) and female (n = 10) neurons. Dotted line represents mean E_{Cl^-} in each sex.

Depolarizing actions of GABAAR activation in males and females

We tested GABAAR evoked changes in membrane potential in perforated patch-clamp configuration and observed that almost all male neurons (15/16 – 94%) responded to muscimol (10 μM) with depolarization, whereas only a few (10/19 – 53%) of female neurons showed depolarization of RMP (Fig. 1B). The sex difference ($\chi^2 = 7.19$; $P < 0.01$) in the number of cells responding with depolarization found here points out that GABAAR activation has greater excitatory actions in males than females. In order to quantify the electrochemical gradient of Cl^- comparatively between sexes we registered the voltage changes (V_Δ) in response to muscimol (10 μM) at various holding potentials (V_h) (from −60 to −20 mV) in perforated patch-clamp configuration. Muscimol pulses cause membrane depolarization at −40, −50 and −60 mV (Fig. 2A), whereas hyperpolarization is observed at more depolarized potentials (−30 and −20 mV). Mean E_{Cl^-} values were −44.67 ± 1.03 and −43.21 ± 2.32 mV, in males and females, respectively. The similarity in E_{Cl^-} between sexes suggests that they share an equal gradient force (Fig. 2B). As may be expected, the difference between males and females in $[Cl^-]_i$ as determined by the Nernst equation, was also not significant (27.61 ± 1.14 and 30.02 ± 2.72 mM, respectively).

When we analysed the voltage changes (mV) and duration time (s) of muscimol evoked responses at various holding potentials, a new sex difference emerged (Fig. 3). Statistical analysis revealed an interaction between sex and holding potential (ANOVA: $F_4 = 5.81$; $P < 0.001$). LSD post hoc test indicated that male neurons present greater mean voltage changes than females at −60 ($P < 0.05$), −30 and −20 mV ($P < 0.001$). Moreover, ANOVA for duration time of responses showed a main effect of sex ($F_1 = 95.23$; $P < 0.001$), male derived cultures presented longer lasting responses compared to females at all holding potentials tested. This evidence supports the existence of greater excitatory GABAAR actions in male hypothalamic neurons that have not been exposed to hormones *in utero*.

GABA sensitivity and current density of GABAAR in male and female neurons

An alternative hypothesis that could explain the sex difference in depolarizing GABAAR actions is a different subunit composition in each sex. There is a very tight relationship among GABA sensitivity and α subunit composition of GABAAR (Böhme *et al.*, 2004; Olsen & Sieghart, 2008). In order to determine GABA sensitivity we performed a dose–response curve in whole-cell voltage clamp configuration to obtain the EC50 value and Hill coefficient for the neurotransmitter in males and females. Even if neurons exhibit a dose dependent response (Fig. 4), there is no sex difference in GABA sensitivity compared by means of EC50 values (males: 24.49 ± 8.54 μM vs. females: 16.04 ± 4.67 μM) and Hill coefficient (males: 1.85 ± 0.31 vs. females: 2.34 ± 0.46), suggesting that males and females share a similar α subunit composition of GABAAR. There were no sex differences in the current amplitude evoked by a saturating dose (500 μM) of GABA (males: 1050.4 ± 193.52 pA vs. females: 1049.71 ± 166.06 pA) nor in the current density of GABAAR ($\delta_{I\text{-}GABAAR}$ = males: 107.19 ± 14.95 pA/pF vs. females: 90.67 ± 21.59 pA/pF).

Discussion

Our findings agree with previous results from other laboratories that have shown more hypothalamic cells with depolarizing responses in males than females *in vivo* (Auger *et al.*, 2001), although authors attributed that difference to a hormonally regulated process mediated by circulating gonadal hormones. Here, we show that male derived cultures have more cells responding with depolarization than female even when they have been taken from embryos at E16, 2 days before neurons would be exposed to gonadal steroids *in utero* and cultured without oestradiol. Moreover, our results show that in neurons with depolarizing responses, males have larger voltage changes (in the order of 10 mV) and longer lasting responses (more than 6 s) than females, suggesting even greater GABAAR actions in male neurons.

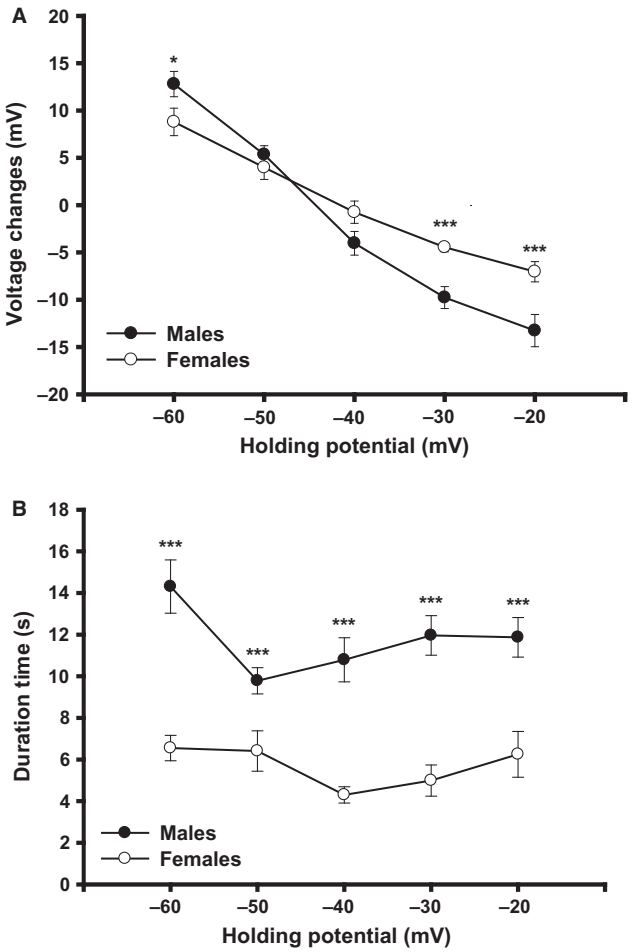

FIG. 3. (A) Voltage changes in membrane potential (mV) and (B) duration time (s) of 10 μM muscimol evoked responses at different holding potentials of male ($n = 15$) and female ($n = 10$) 9 DIV hypothalamic neurons. Values represent mean ± SEM, analysed by two-way ANOVA followed by LSD post hoc test. * $p < 0.05$ males vs. females at -60 mV holding potential, *** $p < 0.001$ males vs. females at -30 and -20 mV holding potentials and in (B) *** $p < 0.001$ males vs. females at all holding potentials.

As the magnitude of depolarizing GABAAR responses is mainly due to a more positive E_{Cl^-} respect to RMP, we determined both values in male and female hypothalamic neurons. Neither parameter differed among male and female neurons, suggesting that Cl^- gradient force is not responsible for the sex difference in GABAAR activation. It has been suggested that gonadal hormones could differentially regulate the chloride co-transporters levels in hypothalamic tissue of embryonic and early postnatal male and female rats and consequently alter Cl^- gradient (Perrot-Sinal *et al.*, 2007). This evidence supports the hypothesis of the gonadal origin of a neural sex difference. In line with this, it has been shown that oestradiol

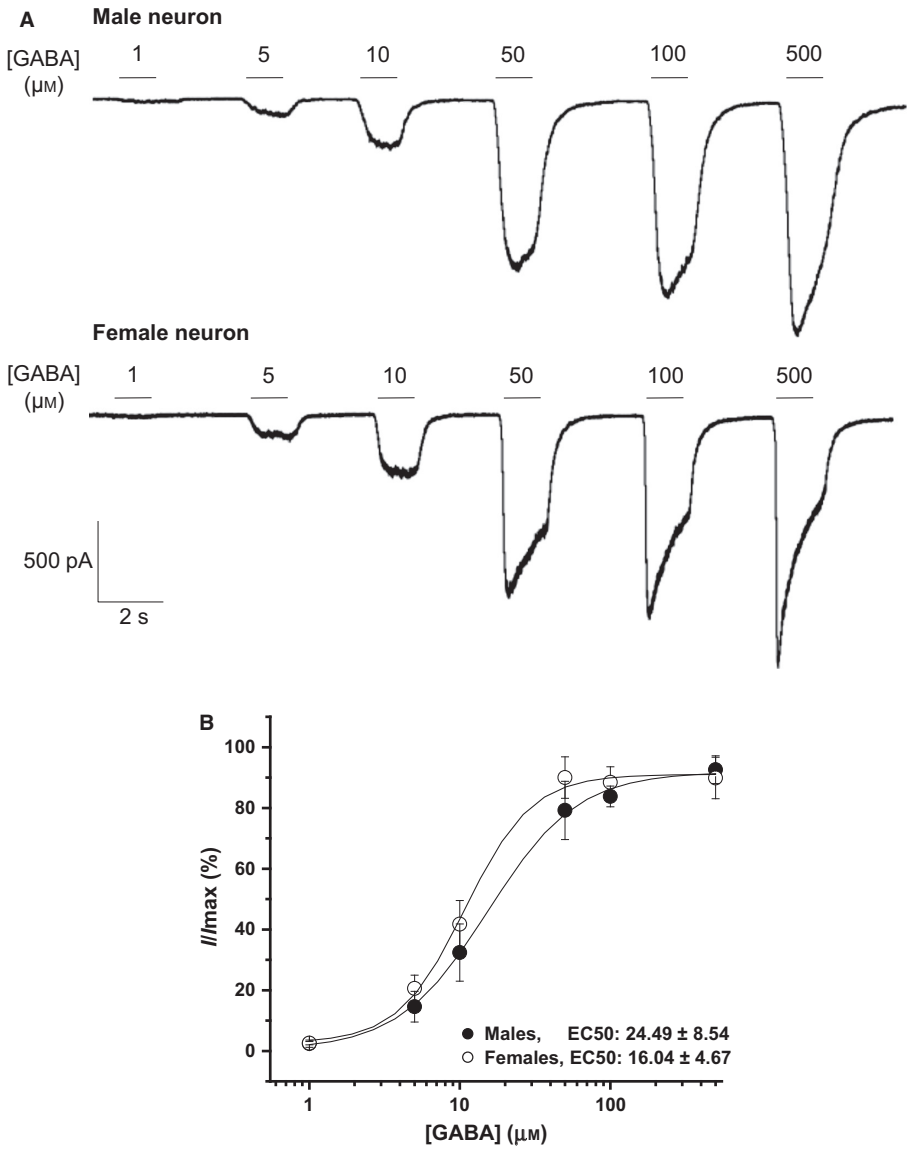

FIG. 4. (A) Representative traces of dose–response currents to GABA (1–500 μM) of a male and a female 9 days *in vitro* (9 DIV) hypothalamic neuron. Bars represent ligand exposure time. (B) dose–response curve to GABA of male (n = 5) and female (n = 7) hypothalamic neurons after 9 DIV. I/I_{max} (%): current percentage respect to maximum current. EC50 values (μM) represent mean ± SEM.

treatment produced amplitude and frequency increases of GABAergic postsynaptic potentials of male derived hypothalamic cultures whereas diminished it in females (Zhou *et al.*, 2005). Oestradiol also increased intracellular calcium levels and extended the time in which muscimol exerts depolarization in mature non-sexually discriminated hypothalamic cultures compared to controls (Perrot-Sinal *et al.*, 2001). Despite this tempting evidence, here we demonstrate that male and female hypothalamic neurons that have not been exposed to any hormonal treatment differ in GABAAR evoked responses.

Although in our cultures hypothalamic neurons had not been exposed to sex hormones, we cannot exclude the possibility of *de novo* synthesis of neurosteroids in hypothalamic neurons, co-cultured glial cells or even by means of the astroglial-conditioned media (Rossetti *et al.*, 2016). We must also consider the possibility of distinct intracellular regulation of GABAAR function in each sex. The mechanism of GABAAR intracellular regulation function is well documented in the literature and includes specific subunit phosphorylation by several kinases, as well as receptor trafficking, clustering and endocytosis by multiple proteins (Connelly *et al.*, 2013; Faheem *et al.*, 2014). However, if this is the case, there must be a sex difference in neurosteroids production or intracellular signalling between male and female hypothalamic neurons that have not been exposed to gonadal hormones, supporting evidence for an intrinsic difference among cells of different sex.

An alternative explanation for the greater magnitude in GABAAR responses in males is a distinct receptor subunits composition between sexes (Olsen & Sieghart, 2008). Sensitivity to GABA, its natural ligand, is determined by which α subunit is present in the receptor (Böhme *et al.*, 2004). However, our results show no sex difference in mean EC50 values or Hill coefficients, strongly suggesting that males and females share, at least a similar α subunit composition of GABAAR. In line with this fact, although there is a developmental switch in GABAAR α subunit expression in hypothalamic regions of rat neonates, there is no evidence of sex differences (Davis *et al.*, 2000). However, we cannot

discard the existence of sex differences in the expression of other GABAAR subunits in embryonic hypothalamic neurons. In fact, in some experimental systems the *in vitro* GABAAR subunit expression pattern recapitulates the *in vivo* pattern (Gao & Fritschy, 1995; Holopainen & Lauren, 2003). It could be possible then, that sex differences in GABAAR function found here show an underling sex-biased subunit conformation of GABAAR. Interestingly, some GABAAR subunits like α3, θ and ε are preferentially co-expressed in embryonic hypothalamic neurons (Pape *et al.*, 2009). Moreover, the gen cluster of α3θε subunits is located on Xq28 chromosome (Korpi *et al.*, 2002). It is well-known that some genes in the X chromosome could escape to X-inactivation (Yang *et al.*, 2010). So a constitutive double X chromosome complement (female neurons in this work) could account for a different α3θε subunits expression level compared with single X chromosome complement carrying cells (males). As receptors that include θ and/or ε subunits exhibit unique functional characteristics (Davies *et al.*, 1997, 2001; Whiting *et al.*, 1997; Bonnert *et al.*, 1999; Ranna *et al.*, 2006), we can assume that a greater expression of θ and/or ε containing GABAAR in female neurons could probably explain the decreased activation of GABAAR found here.

In summary, we have demonstrated that male hypothalamic neurons exhibit larger GABAAR depolarizing responses than female neurons at 9 DIV. We have also shown that sex differences in GABAAR actions are independent of Cl^- gradient or GABA sensitivity suggesting that other factors, not identified here, are responsible for such differences. Most importantly, we showed that these differences in GABAAR function among male and female hypothalamic neurons are independent of gonadal hormones. These results strengthen the need to re-evaluate the role of hormones in the determination of sex differences in the brain.

Author contributions

FRM, HFC and MJC conception and design of research. FRM performed all experiments and analysed data. FRM, HFC and MJC interpreted results of experiments. FRM prepared figures. FRM and MJC wrote manuscript. HFC edited and revised manuscript. MJC drafted manuscript.

Disclosures

None.

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Abbreviations

$[Cl^-]_i$, intracellular Cl^- concentration; C, capacitance; Cl^-, chloride ions; CNS, central nervous system; DIV, days *in vitro*; $E_{Cl}{}^-$, Cl^- reversal potential; E, embryonic day; GABAAR, $GABA_A$ receptor; GABA, γ-aminobutyric acid; PN, postnatal day; R, membrane resistance; RMP, resting membrane potential; VDCC, voltage-dependent calcium channels; $\delta_{I\text{-}GABAAR}$, $GABA_A$ receptor current density.

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