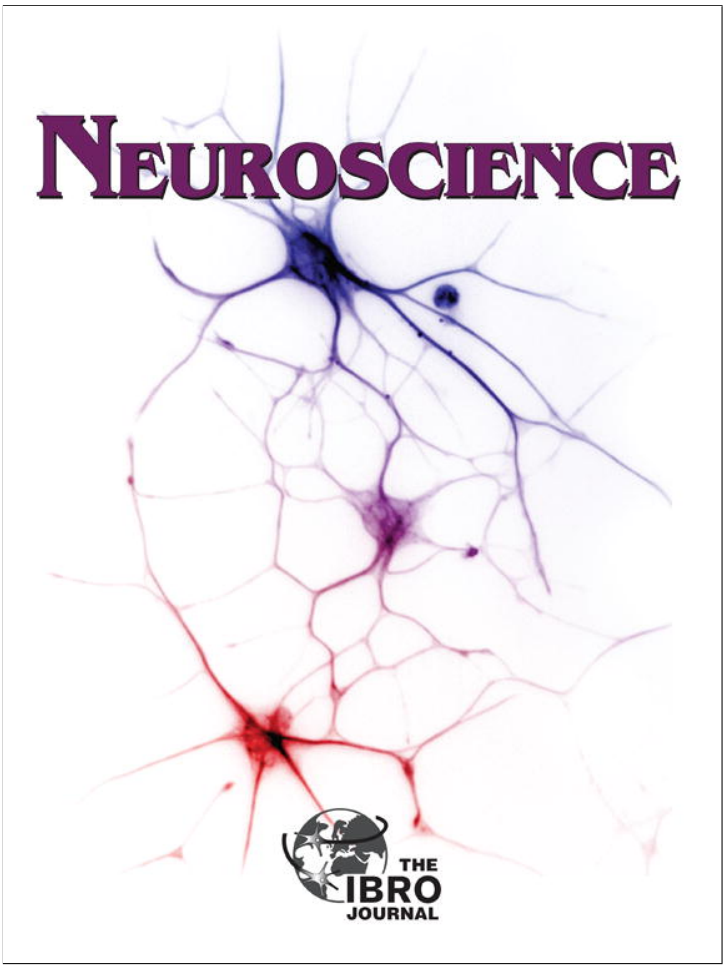

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ALLOPREGNANOLONE AND PUBERTY: MODULATORY EFFECT ON GLUTAMATE AND GABA RELEASE AND EXPRESSION OF 3α-HYDROXYSTEROID OXIDOREDUCTASE IN THE HYPOTHALAMUS OF FEMALE RATS

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Abstract—The hypothalamic release of glutamate and GABA regulates neurosecretory functions that may control the onset of puberty. This release may be influenced by neurosteroids such as allopregnanolone. Using superfusion experiments we examined the role of allopregnanolone on the K^+-evoked and basal [^{3}H]-glutamate and [^{3}H]-GABA release from mediobasal hypothalamus and anterior preoptic area in prepubertal, vaginal opening and pubertal (P) rats and evaluated its modulatory effect on $GABA_A$ and NMDA (*N*-methyl-D-aspartic acid) receptors. Also, we examined the hypothalamic activity and mRNA expression of 3α-hydroxysteroid oxidoreductase (3α-HSOR) – enzyme that synthesizes allopregnanolone – using a spectrophotometric method and RT-PCR, respectively. Allopregnanolone increased both the K^+-evoked [^{3}H]-glutamate and [^{3}H]-GABA release in P rats, being the former effect mediated by the modulation of NMDA receptors – as was reverted by Mg^{2+} and by the NMDA receptor antagonist AP-7 and the latter by the modulation of NMDA and $GABA_A$ receptors – as was reverted by Mg^{2+} and the $GABA_A$ receptor antagonist bicuculline. The neurosteroid also increased the basal release of [^{3}H]-glutamate in VO rats in an effect that was dependent on the modulation of NMDA receptors as was reverted by Mg^{2+}. On the other hand we show that allopregnanolone reduced the basal release of [^{3}H]-GABA in P rats although we cannot elucidate the precise mechanism by which the neurosteroid exerted this latter effect. The enzymatic activity and the mRNA expression of 3α-HSOR were both increased in P rats regarding the other two studied stages of sexual development. These results suggest an important physiological function of allopregnanolone in the hypothalamus of the P rat where it might be involved in the 'fine tuning' of neurosecretory functions related to the biology of reproduction of the female rats.

Key words: puberty, allopregnanolone, glutamate release, GABA release, 3α-hydroxysteroid oxidoreductase, hypothalamus.

INTRODUCTION

Puberty is the phase of the sexual development during which the capacity for reproduction is reached. In female rats it becomes evident in the vaginal opening (VO), when they manifest sexual behavior and estrous cycle-dependent hormonal secretion. At this stage is of pivotal importance the initiation of a pulsatile secretion of the hypothalamic GnRH (gonadotropin-releasing hormone). This hormone drives the synthesis and release of gonadotropins from the pituitary that is required for fertility (Sarkar et al., 1976). In the evening of the proestrus, the activity of GnRH neurons switches from a pulsatile to a surge mode of secretion that initiates ovulation. During the pubertal (P) development this surge mode appears to be the consequence of an acceleration in the GnRH pulse frequency as the age increases (Sisk et al., 2001). It is well-known that estradiol and progesterone are the main regulators of these cyclical changes in the GnRH secretion through feedback actions (Chappell and Levine, 2000; Micevych et al., 2003). The initiation of puberty is also associated to the release of neurotransmitters from afferent inputs that activate GnRH neurons in the hypothalamus (Insel et al., 1990; Ojeda et al., 2003). Several neurotransmitters and neuropeptides regulate the activity of these neurons in puberty and adulthood, being among the most important, glutamate (Urbanski and Ojeda, 1987; Brann and Mahesh, 1997), GABA (Clarkson and Herbinson, 2006), neuropeptide Y (Wójcik-Gładysz and Polkowska, 2006), and kisspeptin (Terasawa et al., 2010; Navarro, 2012). Additionally, the release of these molecules may be regulated by a great number of factors that act on the

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Abbreviations: 3α-HSOR, 3α-hydroxysteroid oxidoreductase; 5α-DHP, 5α-hydroxysteroid reductase; Allo, allopregnanolone-3α-hydroxy-5α-pregnan-20-one; ANOVA, analysis of variance; AP-7, 2-amino-7-phosphonoheptanoic acid; APOA, anterior preoptic area; Bic, bicuculline; dNTPs, deoxynucleoside triphosphates; EDTA, ethylenediaminetetraacetic acid; [^{3}H]-Glu, [^{3}H]-glutamic acid; GnRH, gonadotropin-releasing hormone; KRBG, Krebs Ringer bicarbonate glucose; MBH, mediobasal hypothalamus; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate; NMDA, *N*-methyl-D-aspartic acid; P, pubertal; PP, prepubertal; Veh, vehicle; VO, vaginal opening; vs., versus.

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hypothalamus, including the above-mentioned estradiol and progesterone (Micevych et al., 2010), leptin (Xu et al., 2012), ghrelin (Fernández-Fernández et al., 2005), and neurosteroids (Zheng, 2009). Even though the advances in this field, the mechanisms by which many of these factors function remain unresolved.

Allopregnanolone-3α-hydroxy-5α-pregnan-20-one-(Allo), a progesterone derivative (Robel and Baulieu, 1994), is one of the best characterized neurosteroids that regulates the function of GnRH neurons (El-Etr et al., 1995; Sim et al., 2001; Giuliani et al., 2011). Its biosynthesis begins with progesterone, which is converted to dihydroprogesterone by the enzyme 5α-hydroxysteroid reductase (5α-DHP) and after that, the enzyme 3α-hydroxysteroid oxidoreductase (3α-HSOR) catalyses the reduction of dihydroprogesterone toward allopregnanolone (Corpéchot et al., 1993). Little is known about the hypothalamic expression of 3α-HSOR, in particular related to the P development although allopregnanolone has been been implicated in neurochemical and neuroendocrine functions such as the modulation of LH (Akwa et al., 1999), GnRH, glutamate (Giuliani et al., 2011) and GABA release (Uchida et al., 2002). Fluctuating serum levels of this neurosteroid have been described across the estrous cycle (Corpéchot et al., 1993; Corpéchot et al., 1997) and also in girls with precocious pubarche (Iughetti et al., 2005). This background prompts us to consider allopregnanolone as a candidate to act as a hypothalamic modulator of the activity of key molecules during the initiation of puberty.

Most of the effects of allopregnanolone appear to be mediated by the interaction of the neurosteroid with neurotransmitter receptors. It is well known that allopregnanolone acts as a potent enhancer of the $GABA_A$ receptor function. At nM concentrations, it facilitates the chloride channel opening by allosteric modulation and therefore, increases the response to the neurotransmitter GABA in neurons that express this subtype of receptor (Harrison et al., 1987; Haage and Johansson, 1999; Shu et al., 2004). At higher concentrations (μM) allopregnanolone may directly open and activate the $GABA_A$ receptor chloride channel (Belelli and Lambert, 2005). On the other hand, the interaction of allopregnanolone with other neurotransmitter receptors, such as glutamate receptors, has not been fully demonstrated. Other neurosteroids have been shown to interact with NMDA (*N*-methyl-D-aspartic acid) receptors such as pregnanolone sulfate (Kussius et al., 2009), dehydroepiandrosterone sulfate and allopregnanolone sulfate (Johansson and Le Grevès, 2005). We have previously reported NMDA receptor-dependent effects of allopregnanolone on the GnRH release, glutamate release (Giuliani et al., 2011) and dopamine release (Cabrera et al., 2002) in adult rats, thus we hypothesize a possible interaction between the neurosteroid and this receptor.

Since we hypothesize that allopregnanolone is an important modulator of the P development, in this study we aim (1) to determine whether the mRNA expression and enzymatic activity of 3α-HSOR are differential in mediobasal hypothalamus (MBH) and anterior preoptic area (APOA) of prepubertal (PP) and P rats as well as in rats undergoing VO. Additionally, keeping in mind the significance that glutamate and GABA have during the sexual development, and the possible role of allopregnanolone modulating their activity we aim (2) to determine whether allopregnanolone could affect the glutamate and GABA release in MBH–APOAs of rats in the three above-mentioned stages of sexual development and (3) to determine whether these possible effects could be due to the modulation of NMDA and $GABA_A$ receptors.

We demonstrate that the hypothalamic mRNA and the enzymatic activity of 3α-HSOR increase as the female rats develop from PP to P stage. Additionally, testing both the basal and K^+-evoked release of [^{3}H]-glutamate and [^{3}H]-GABA from MBH–APOA explants we have found that allopregnanolone modulates the GABA and glutamate release and that these effects could involve an interaction of the neurosteroid with NMDA and $GABA_A$ receptors.

EXPERIMENTAL PROCEDURES

Animals

Sprague–Dawley female rats (30–52 days old) from our laboratory colony were used. They were maintained at a constant temperature (22 °C ± 2 °C) and lighting (lights on between 07.30–19.30 h) and were housed in groups (3 animals/cage) with free access to standard rat chow and tap water. All the animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23). The animals used in this study were (1) PP rats (30 days old); (2) VO rats (36–40 days old); or (3) P rats (48–52 days old). Rats belonging the latter group were selected on the afternoon of the proestrus (16:00 h) when the LH surge had not yet started.

Chemicals

The neurosteroids allopregnanolone and dihydroprogesterone (substrate of 3α-HSOR), the reduced cofactor NADPH, EDTA, the competitive NMDA receptor antagonist, 2-amino-7-phosphonoheptanoic acid (AP-7) and the competitive $GABA_A$ receptor antagonist bicuculline ((−)-bicuculline methiodide, 1(S), 9(R)) (Bic) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Glutamic acid, L-[3,4-^{3}H] ([^{3}H]-Glu) and gamma aminobutyric acid-[2, 3-^{3}H (N)] ([^{3}H]-GABA) were purchased from New England Nuclear, Boston, MA, USA. TRIZOL reagent, sense and antisense 3α-HSOR primers were from Invitrogen Life Technologies, Buenos Aires, BA, Argentina. Deoxynucleoside triphosphates (dNTPs), MMLV Reverse Transcriptase and Go Taq DNA polymerase were from Promega Inc., Madison, WI, USA. Hexameric Random Primers were from Biodynamics S.R.L., Buenos Aires, BA, Argentina.

Drugs preparation

Allopregnanolone was initially dissolved in propylenglycol to a concentration of 0.6 mM. The further 120 nM concentration of allopregnanolone was obtained by dilution in Krebs Ringer bicarbonate glucose (KRBG) Mg^{2+}-free buffer at pH 7.4 (118.6 mM NaCl, 4.75 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2P0_4, 1.2 mM Na_2S0_4, 1.2 mM $NaHC0_3$, 5.5 mM dextrose and 0.06 mM ascorbic acid, saturated with 95% O_2/5% CO_2). This concentration was chosen based on previous results of our laboratory indicating neurochemical $GABA_A$ receptor-dependent

effects of allopregnanolone in the striatum (Cabrera et al., 2002). In addition, we considered that the neurosteroid allosterically modulates $GABA_A$ receptor in the nanomolar range (Harrison et al., 1987; Haage and Johansson, 1999; Shu et al., 2004).

The 9.8 μM concentration of Bic and the 100 μM concentration of AP-7 were obtained by dilution in KRBG–Mg^{2+}-free buffer at pH 7.4 as it has been described by Donoso et al. (1992) and in our previous report (Giuliani et al., 2011) respectively. As Mg^{2+} ions are natural blockers of NMDA receptors, in the experiments aimed to study the blockade of NMDA receptors under physiological conditions, KRBG buffer was prepared using 1.2 mM $MgSO_4$ instead of Na_2SO_4 (pH 7.4) as it has been assayed by Musante et al. (2011). As a control, KRBG–Mg^{2+}-free buffer at pH 7.4 with propylene glycol as vehicle (Veh) were used.

Explants dissection

The animals were killed by decapitation at 16:00 h, their brains rapidly removed and cooled on ice and the MBH–APOA explants dissected out. The anterior border of each block of tissue was made by a coronal cut just anterior to the entry point of the optic chiasm and the posterior border by a coronal cut just behind the pituitary stalk. The lateral limits were the hypothalamic fissures and the in-depth limit was the subthalamic sulcus.

RNA isolation and multiplex RT-PCR analysis of 3α-HSOR

MBH/APOAs of PP ($n = 6$), VO ($n = 6$) and P ($n = 6$) rats were dissected and the total RNA isolated using TRIZOL reagent, according to the manufacturer's instructions. The gel electrophoresis and ethidium bromide staining confirmed the integrity of the samples. The quantification of the RNA was based on the spectrophotometric analysis at 260 nm. Two micrograms of total RNA were retro-transcribed with 200 U of MMLV Reverse Transcriptase, using random primer hexamers in a 50 μl reaction mixture following the manufacturer's instructions. The fragments coding for rat 3α-HSOR and rat cyclophilin (as endogenous control) were amplified by multiplex PCR with specific primers for 3α-HSOR (forward: 5′-CAAGTGCCTTTGAATGCTGA-3′; and reverse: 5′-CCTGGAGCTCTGGTTCTTGG-3′) and rat cyclophilin (forward: 5′-CAAGACTGAGTGGGTGGATG G-3′ and reverse: 5′-ACTTGAAGGGGAATGAGGAAA-3′), in a reaction mixture containing 5× Go Taq reaction buffer, 0.2 mM dNTPs, 0.6 μl (5 μM) 3α-HSOR primers, 0.3 μl (5 μM) cyclophilin primers, 0.3 μl Go Taq DNA polymerase (Promega Inc.) and 7 μl RT-generated cDNA in a 25 μl final reaction volume. The predicted sizes of the PCR-amplified products were 379 bp for 3α-HSOR and 293 bp for cyclophilin. The PCR products were electrophoresed on 2% agarose gels, visualized with ethidium bromide (5.5 mg/ml), and examined by ultra-violet transillumination. The band intensities of the PCR products were quantified using Image J (Image Processing and Analysis from http://rsb.info.nih.gov/ij) and expressed as arbitrary units of 3α-HSOR relative to cyclophilin.

3α-HSOR enzymatic activity assay

MBH/APOAs isolated from PP ($n = 7$), VO ($n = 6$) and P ($n = 6$) rats were homogenized in 2 ml of ice-cold 10 mM phosphate buffer (pH 6.5) containing 0.154 M KCl, 1 mM dithiothreitol, 0.5 mM EDTA, and 1 M PMSF. The homogenate was centrifuged at 105000*g* for 60 min at 4 °C in an ultracentrifuge with a T 40.2 rotor, Beckman Model L (Palo Alto, CA, USA). The supernatant fractions (cytosolic fractions) were stored at −80 °C until the enzymatic activity assay and the quantitative analysis. The enzymatic activity assay was carried out as described Takahashi et al. (1995) with minor modifications (Escudero et al., 2012). Briefly, the 3α-HSOR activity from each cytosolic fraction was determined spectrophotometrically by measuring the oxidation rate of NADPH at 340 nm and 37 °C in a 1.00 cm-pathlength cuvette with a Metrolab 1600 DR (USA) spectrophotometer. The reductase activity was measured in 100 mM phosphate buffer (pH 6.5) containing 0.1 mM NADPH, 0.08 mM 5α-DHP (substrate), and enzyme solution (100 μl, cytosolic fraction) in a total volume of 1.0 ml. The reaction was initiated by the addition of the cofactor NADPH to the assay mixture. A blank without substrate was included. The protein concentration for each cytosolic fraction was determined by the Lowry's method using bovine serum albumin as a standard. The 3α-HSOR activity was expressed as nmol of substrate consumed per milligram of protein per minute.

Superfusion experiments

Each MBH–APOA dissected from the brain of a PP, VO or P rat was longitudinally sliced at 240 μm with a McIlwain tissue chopper (The Mickle Lab Eng Co. Ltd. Cat: 6571). Depending on whether the experiment was carried out to study the glutamate or GABA release, each set of slices obtained per MBH–APOA explant was respectively exposed to 2 μl [^{3}H]-Glu (specific activity 49.6 Ci/mmol) or 2 μl [^{3}H]-GABA (specific activity 76.2 Ci/mmol) diluted in 2 ml of gassed (95% O_2 and 5% CO_2) KRBG–Mg^{2+}-free buffer for 15 min at 37 °C in a Dubnoff metabolic shaker (Precision Scientific Group. Cat: 66722). After that, the slices were transferred to superfusion chambers and superfused at 0.7 ml/min with KRBG–Mg^{2+}-free buffer for 30 min (washing period), to washout the [^{3}H]-neurotransmitter not incorporated into the tissue. Then, the experiments were started by superfusing KRBG–Mg^{2+}-free buffer containing either the Veh or 120 nM allopregnanolone (pre-stimulus period). During this period, five fractions of 1.75 ml each (2.5 min per fraction) were collected and considered as basal release. After that, the slices were superfused with KRBG Mg^{2+} free supplemented with 28 mM KCl and three 1.75 ml fractions were collected (K^+-evoked release). Then, the same solution than the pre-stimulus period, was superfused and five 1.75 ml fractions were collected. At the end of the experiments, the slices were homogenized in 2 ml of 0.2 M perchloric acid by sonication, and 0.5 ml aliquots of each fraction and homogenate were taken and mixed with scintillation fluid to measure the radioactivity.

The experiments that were conducted to determine the modulatory effects of allopregnanolone on NMDA and $GABA_A$ receptors were carried out on MBH–APOAs of P rats as they were demonstrated to be responsive to the neurosteroid. To antagonize NMDA receptors under physiological conditions, in the pre- and post-stimulus periods 120 nM allopregnanolone diluted in KRBG–Mg^{2+} buffer (Mg^{2+} + Allo groups) or KRBG–Mg^{2+} buffer alone (Mg^{2+} groups) was used for both [^{3}H]-glutamate release and [^{3}H]-GABA release experiments. To antagonize $GABA_A$ receptors, 120 nM allopregnanolone plus 9.8 μM Bic (Bic + Allo groups) or 9.8 μM Bic alone (Bic groups) was used. Moreover, in [^{3}H]-glutamate release experiments, 120 nM allopregnanolone co-administered with 100 μM AP-7 (AP-7 + Allo group) or 100 μM AP-7 alone (AP-7 group), was also assayed to antagonize NMDA receptors. To ensure that NMDA and $GABA_A$ receptors were blocked before the superfusion with Allo + Mg^{2+}, Allo + AP-7, or Allo + Bic solutions, the tissues were superfused with the antagonists alone (KRBG–Mg^{2+}, 100 μM AP-7 or 9.8 μM Bic, respectively), five minutes before starting collecting fractions.

The number of animals per treatment group in [^{3}H]-glutamate release experiments were: PP rats-Veh ($n = 5$), PP rats-Allo ($n = 6$), VO rats-Veh ($n = 9$), VO rats-Allo ($n = 8$), P rats-Veh ($n = 11$), P rats-Allo ($n = 13$), P rats-Mg^{2+} + Allo ($n = 10$), P rats-Mg^{2+} ($n = 6$), P rats-Bic + Allo ($n = 8$), P rats-Bic ($n = 5$).

The number of animals per treatment group in [^{3}H]-GABA release experiments were: PP rats-Veh (n = 5), PP rats-Allo (n = 6), VO rats-Veh (n = 6), VO rats-Allo (n = 7), P rats-Veh (n = 9), P rats-Allo (n = 10), P rats-Mg^{2+} + Allo (n = 6), P rats-Mg^{2+} (n = 9), P rats-Bic + Allo (n = 7), P rats-Bic (n = 5).

Calculations of release data

The amount of radioactivity released in each fraction was expressed as a per cent of the total tritium tissue content at the start of the respective collection period (% [^{3}H]-Glu or [^{3}H]-GABA release). The basal neurotransmitter release was calculated as the average of the % neurotransmitter released from fractions No. 3, 4 and 5. The average of the % neurotransmitter released from fractions No. 6, 7 and 8 was considered as stimulated release and expressed as a percentage of the basal release [(Mean of fractions No. 6, 7, 8/ Mean of fractions No. 3, 4, 5) × 100]. The net percentage of K^+-evoked neurotransmitter release was estimated by subtracting the basal release (considered as 100%) from the stimulated release.

Statistics

All data are presented as means ± SEM. We performed the test of Shapiro–Wilks in order to determine if our data came from normally distributed populations.

For comparisons among the different stages of the sexual development in the superfusion experiments (net K^+-evoked neurotransmitter release and basal neurotransmitter release), two-way analysis of variance (2-way ANOVA) was used in a 3 × 2 factorial design, in which the stages of the sexual development (PP, VO or P) and the experimental conditions (Veh or Allo) were the factors. For comparisons of the net K^+-evoked neurotransmitter release among the different experimental conditions in P rats, 1-way ANOVA was used. The analysis of the different profiles of neurotransmitter release was carried out by using 2-way ANOVA in a 2 × 6 factorial design, in which the experimental conditions (Veh or Allo) and the considered fractions (3–8) were the factors. For comparisons among the different stages of the sexual development in the multiplex PCR and enzymatic experiments, 1-way ANOVA was used. Each statistical analysis was followed by the post hoc Bonferroni test. Differences of $p < 0.05$ were considered statistically significant.

RESULTS

Differential mRNA expression of 3α-HSOR

There was an increased mRNA expression of 3α-HSOR in the MBH–APOAs of P rats compared with PP rats (71.75 ± 3.91% vs. 52.70 ± 3.80%, $p < 0.05$). Despite the lack of statistical differences between VO and PP rats (VO rats: 57.42 ± 6.14%) and between P and VO rats we also observed a slightly increased mRNA expression tendency as rats developed from pre-puberty to puberty (Fig. 1).

Differential enzymatic activity of 3α-HSOR

The enzymatic activity of 3α-HSOR in MBH–APOAs was increased in P rats compared with PP rats [16.45 ± 2.78 vs. 5.24 ± 1.48 (nmol/mg prot min), $p < 0.01$]. Moreover, the activity was slightly increased in P rats compared with VO rats (VO rats: 10.26 ± 1.88 nmol/mg prot min) and in VO rats compared with PP rats although these differences were not statistically significant ($p > 0.05$) (Fig. 2).

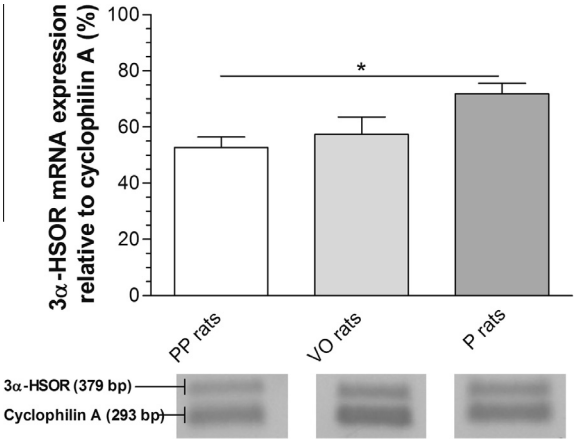

Fig. 1. Differential 3α-HSOR mRNA expression in MBH/APOAs of prepubertal (PP), vaginal opening (VO) and pubertal (P) rats. The columns represent the mean ± SEM of relative units obtained from each group. $^*p < 0.05$. Lower panel: representative bands after electrophoresis and ethidium bromide dying of amplified products.

Effect of allopregnanolone on the [^{3}H]-glutamate release

The exposure of the MBH–APOA slices to allopregnanolone significantly enhanced the net K^+-evoked [^{3}H]-Glu release in P rats (22.55 ± 2.21% vs. 39.85 ± 4.60%, Veh vs. Allo; $p < 0.01$) [$F = 3.25$; DFn = 2; DFd = 49; $p = 0.0475$ (for experimental condition factors) and $F = 7.72$; DFn = 2; DFd = 49; $p = 0.0012$ (for stage of sexual development factors)]. Although there was a trend of increase in the Veh-treated group of VO rats compared with the other two analyzed ages, these differences were not statistically significant ($p > 0.05$) (Fig. 3a).

The effect of the neurosteroid could also be appreciated in the graphs of [^{3}H]-Glu release profiles (Fig. 3b, c). The 2-way ANOVA revealed that the main source of variation in the profiles was the treatment with allopregnanolone [$F = 17.28$; DFn = 5; DFd = 260; $p < 0.0001$ (for experimental condition factors) and

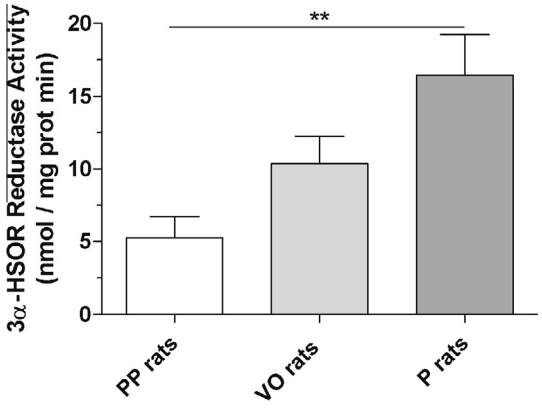

Fig. 2. Differential 3α-HSOR enzymatic activity in MBH/APOA of prepubertal (PP), vaginal opening (VO) and pubertal (P) rats. Results are expressed as mean ± SEM of nmol/mg prot min. $^{**}p < 0.01$.

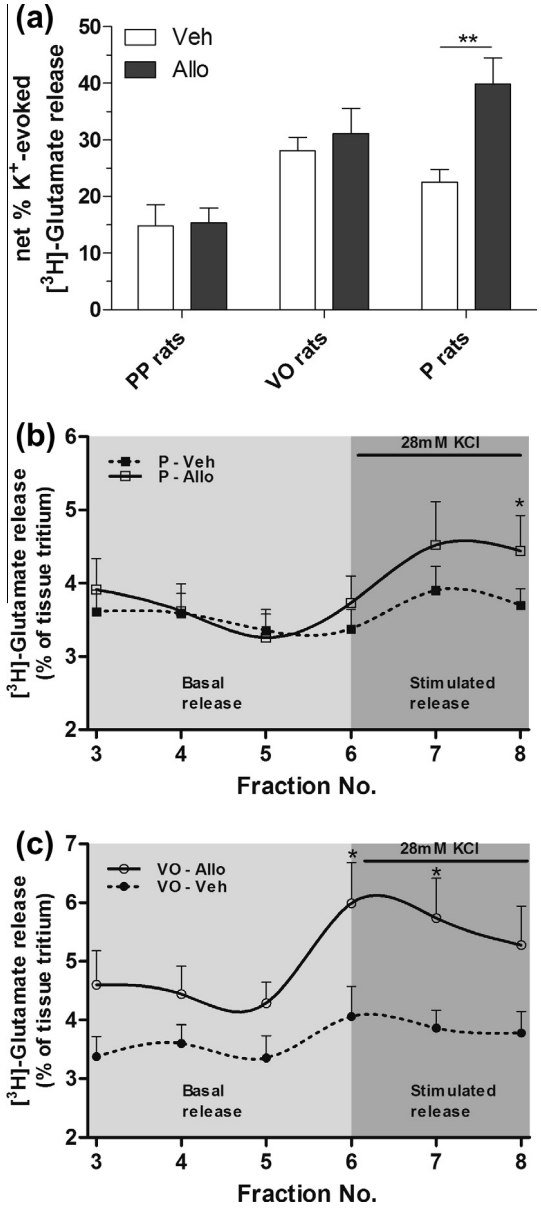

Fig. 3. (a) Effect of allopregnanolone on the net % K^+-evoked [^{3}H]-Glu release through the sexual development *in vitro* (mean ± SEM); $^{**}p < 0.01$. (b, c) Profiles of [^{3}H]-Glu release from MBH–APOAs of pubertal and vaginal opening rats respectively (mean ± SEM of % tissue tritium). The slices were superfused with KRBG containing either Veh (dashed line) or 120 nM Allo (solid line). In the stimulated release period the KRBG buffer was supplemented with 28 mM KCl as a depolarizing stimulus to evoke the neurotransmitter release. Statistical differences between equivalent fractions are labeled with asterisks; $^{*}p < 0.05$.

$F = 2.04$; DFn = 5; DFd = 260; $p = 0.0740$ (for fraction factors)]. In MBH–APOAs of P rats, the superfusion with allopregnanolone induced a trend of increase in the [^{3}H]-Glu release in the stimulated release period these differences being statistically significant in the fraction No. 8 (3.70 ± 0.23% vs. 5.05 ± 0.58%, Veh vs. Allo; $p < 0.05$) (Fig. 4b). In turn in VO rats, the neurosteroid induced a general increase in the [^{3}H]-Glu released from MBH–APOAs in all the analyzed fractions. These differences were statistically significant in the fractions No. 6 (5.99 ± 0.70% vs. 4.06 ± 0.51%, Veh vs. Allo; $p < 0.05$) and No. 7 (5.74 ± 0.68% vs. 3.86 ± 0.30%, Veh vs. Allo; $p < 0.05$) (Fig. 4c). The superfusion with allopregnanolone in MBH–APOAs of PP rats did not induce any change in the profiles of [^{3}H]-Glu release ($p > 0.05$; data not shown).

Effect of allopregnanolone on the [^{3}H]-GABA release

The exposure of MBH–APOA slices to allopregnanolone significantly enhanced the net K^+-evoked [^{3}H]-GABA release in P rats (30.89 ± 4.53% vs. 55.60 ± 7.33%, Veh vs. Allo; $p < 0.01$) [$F = 5.19$; DFn = 1; DFd = 37;

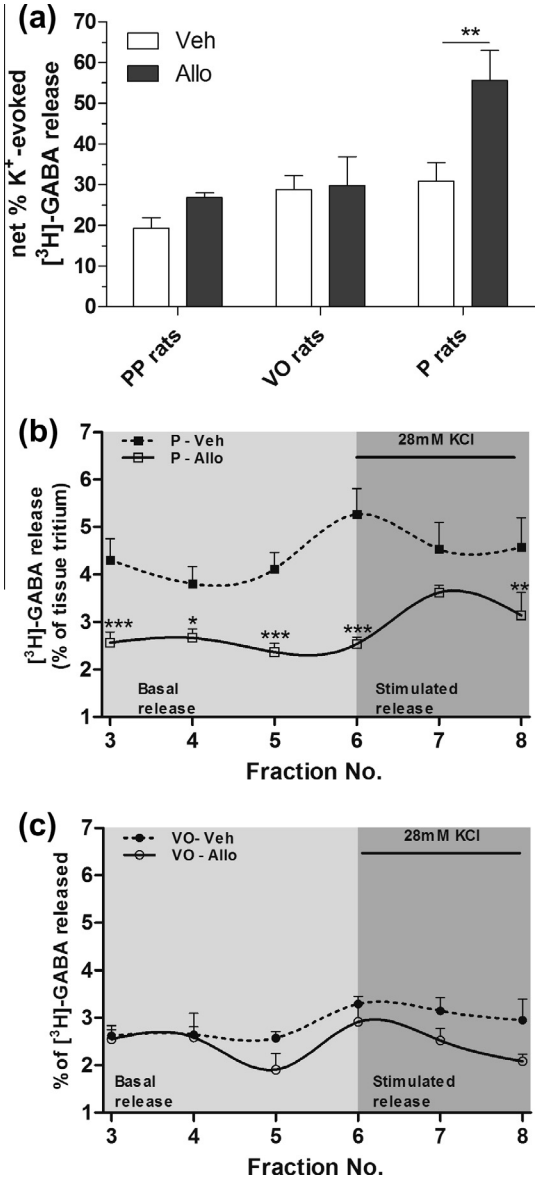

Fig. 4. (a) Effect of allopregnanolone on the net % K^+-evoked [^{3}H]-GABA release through the sexual development *in vitro* (mean ± SEM); $^{**}p < 0.01$. (b, c) Profiles of [^{3}H]-GABA release from MBH–APOAs of pubertal and vaginal opening rats respectively (mean ± SEM of % tritium). The slices were superfused with KRBG containing either Veh (dashed line) or 120 nM Allo (solid line). Statistical differences between equivalent fractions are labeled with asterisks; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

$p = 0.0286$ (for experimental condition factors) and $F = 6.68$; DFn = 2; DFd = 37; $p = 0.0033$ (for stage of the sexual development factors)] (Fig. 4a).

Even though this difference exists, the graphs of [^{3}H]-GABA release profiles showed that allopregnanolone induced an overall decrease in the [^{3}H]-GABA release in both the basal and the stimulated release period (Fig. 4b, c). The 2-way ANOVA confirmed that the main source of variation was the treatment with the neurosteroid [$F = 40.64$; DFn = 5; DFd = 207; $p < 0.0001$ (for experimental condition factors) and $F = 3.07$; DFn = 5; DFd = 207; $p = 0.0107$ (for fraction factors)]. In MBH–APOAs of P rats the differences were statistically significant in the fractions No. 3 (4.31 ± 0.45% vs. 3.57 ± 0.22%, Veh vs. Allo; $p < 0.001$); No. 4 (3.80 ± 0.37% vs. 2.67 ± 0.19%, Veh vs. Allo; $p < 0.05$); No. 5 (4.12 ± 0.35% vs. 2.37 ± 0.18%, Veh vs. Allo; $p < 0.001$); No. 6 (5.27 ± 0.54% vs. 2.54 ± 0.15%, Veh vs. Allo; $p < 0.001$) and No. 8 (4.58 ± 0.63% vs. 3.14 ± 0.48%, Veh vs. Allo; $p < 0.01$) (Fig. 4b). In VO rats the neurosteroid induced a trend of decrease in the [^{3}H]-GABA release but the differences were not statistically significant ($p < 0.05$) (Fig. 4c). The superfusion with allopregnanolone in MBH–APOAs of PP rats did not induce statistically significant changes in the profiles of [^{3}H]-GABA release ($p > 0.05$; data not shown).

Effect of allopregnanolone on the net K^+-evoked [^{3}H]-glutamate release through neurotransmitter receptors modulation

The blockade of NMDA receptors with Mg^{2+} reversed the stimulatory action of allopregnanolone on the net K^+-evoked [^{3}H]-Glu release (39.85 ± 4.60% vs. 18.00 ± 2.50%, Allo vs. Mg^{2+} + Allo; $p < 0.001$). A similar effect had the antagonism of NMDA receptors with AP-7 (39.85 ± 4.60% vs. 22.25 ± 2.52%, Allo vs. AP-7 + Allo; $p < 0.01$) (Fig. 5a). The graphs of the release profiles showed that both magnesium and AP-7 attenuated the effect of the neurosteroid although the differences between the analyzed fractions were not statistically significant ($p > 0.05$) (Fig. 5b). Neither magnesium nor AP-7 administered alone had per se effects on the net K^+-evoked [^{3}H]-Glu release compared to the Veh group ($p > 0.05$ in each case). On the other hand the antagonism of $GABA_A$ receptors with Bic did not change the effect of allopregnanolone on the K^+ evoked [^{3}H]-Glu release (39.85 ± 4.60% vs. 35.31 ± 4.46%, Allo vs. Bic + Allo; $p > 0.05$) (Fig. 5c). This trend could also be observed in the graphs of the profiles of glutamate release (Fig. 3d). Bic alone had no per se effect on the net K^+-evoked [^{3}H]-Glu release compared to the Veh group ($p > 0.05$).

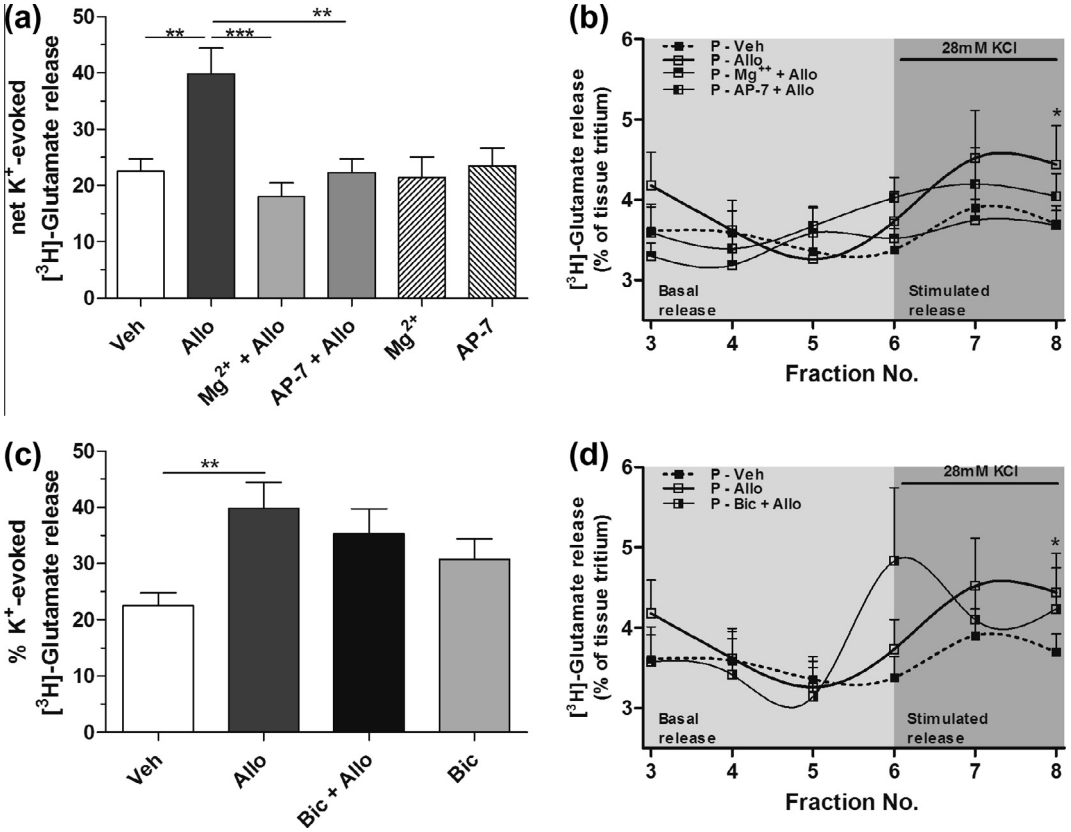

Fig. 5. Antagonism of NMDA and $GABA_A$ receptors and the effect of allopregnanolone on the [^{3}H]-Glu release in MBH–APOAs of pubertal rats. (a) The blockade of NMDA receptors with KRBG–Mg^{2+} and the antagonism with AP-7 revert the effect of 120 nM Allo on the net K^+-evoked [^{3}H]-Glu release; (mean ± SEM) $^{**}p < 0.01$; $^{***}p < 0.001$. (c) The antagonism of $GABA_A$ receptors by application of Bic does not change the effect of 120 nM Allo (mean ± SEM) $^{**}p < 0.01$. The respective profiles of [^{3}H]-Glu release are shown in (b, d); (mean ± SEM) $^{*}p < 0.05$ (Veh vs. Allo).

Effect of allopregnanolone on K^+-evoked [^{3}H]-GABA release through neurotransmitter receptors modulation

The blockade of NMDA receptors with Mg^{2+} reversed the stimulatory action of allopregnanolone on the net K^+-evoked [^{3}H]-GABA release (55.60 ± 7.33% vs. 22.00 ± 7.93%; Allo vs. Mg^{2+} + Allo, $p < 0.01$) (Fig. 6a). Mg^{2+} alone had no per se effect compared to the Veh group ($p > 0.05$). The graph of the profiles of release showed that even though magnesium attenuated the effect that allopregnanolone had in the stimulated release period, it did not change the overall reduced effect that the neurosteroid had on GABA release (Fig. 6b). The differences compared with the control group were significant between the fractions No. 3 (4.31 ± 0.45 vs. 2.65 ± 0.30, Veh vs. Mg^{2+} + Allo; $p < 0.01$), No. 5 (4.12 ± 0.35 vs. 2.65 ± 0.28, Veh vs. Mg^{2+} + Allo; $p < 0.01$), No. 6 (5.27 ± 0.54 vs. 2.74 ± 0.30, Veh vs. Mg^{2+} + Allo; $p < 0.001$) and No. 8 (4.58 ± 0.63 vs. 2.80 ± 0.33, Veh vs. Mg^{2+} + Allo; $p < 0.05$) (Fig. 6b).

The antagonism of $GABA_A$ receptors with Bic reversed the effect of the neurosteroid on net K^+-evoked [^{3}H]-GABA release (55.60 ± 7.33% vs. 26.00 ± 4.53%, Allo vs. Bic + Allo; $p < 0.01$) (Fig. 6c). Bic alone had no per se effect on [^{3}H]-GABA release ($p > 0.05$). However, the graph of the profiles of release showed that Bic did not change the overall reduced effect that the neurosteroid had on the GABA release (Fig. 6d). The differences compared with the control group were significant between the fractions No. 1 (4.31 ± 0.45% vs. 2.70 ± 0.25, Veh vs. Bic + Allo; $p < 0.05$), No. 6 (5.27 ± 0.54% vs. 3.23 ± 0.29%, Veh vs. Bic + Allo; $p < 0.001$) and No. 8 (4.58 ± 0.63% vs. 2.88 ± 0.24%, Veh vs. Bic + Allo, $p < 0.05$) (Fig. 6d).

Effect of allopregnanolone on the basal [^{3}H]-glutamate and [^{3}H]-GABA release

To confirm the tendencies observed in the profiles of release we analyzed the averages of basal [^{3}H]-Glu and [^{3}H]-GABA release. The addition of allopregnanolone to the superfusion media induced an increase in the average basal [^{3}H]-Glu release in VO rats but did not induce any significant change in the other two analyzed ages (Table 1). The 2-way ANOVA revealed that the main source of variation was the sexual development stage [$F = 5.58$; DFn = 1; DFd = 46; $p = 0.0068$ (for stage of sexual development factors) and $F = 0.73$; DFn = 1; DFd = 46; $p = 0.3981$(for experimental condition factors)]. The blockade of NMDA receptors with the addition of Mg^{2+} in the superfusion medium induced a slight reduction in the average basal release

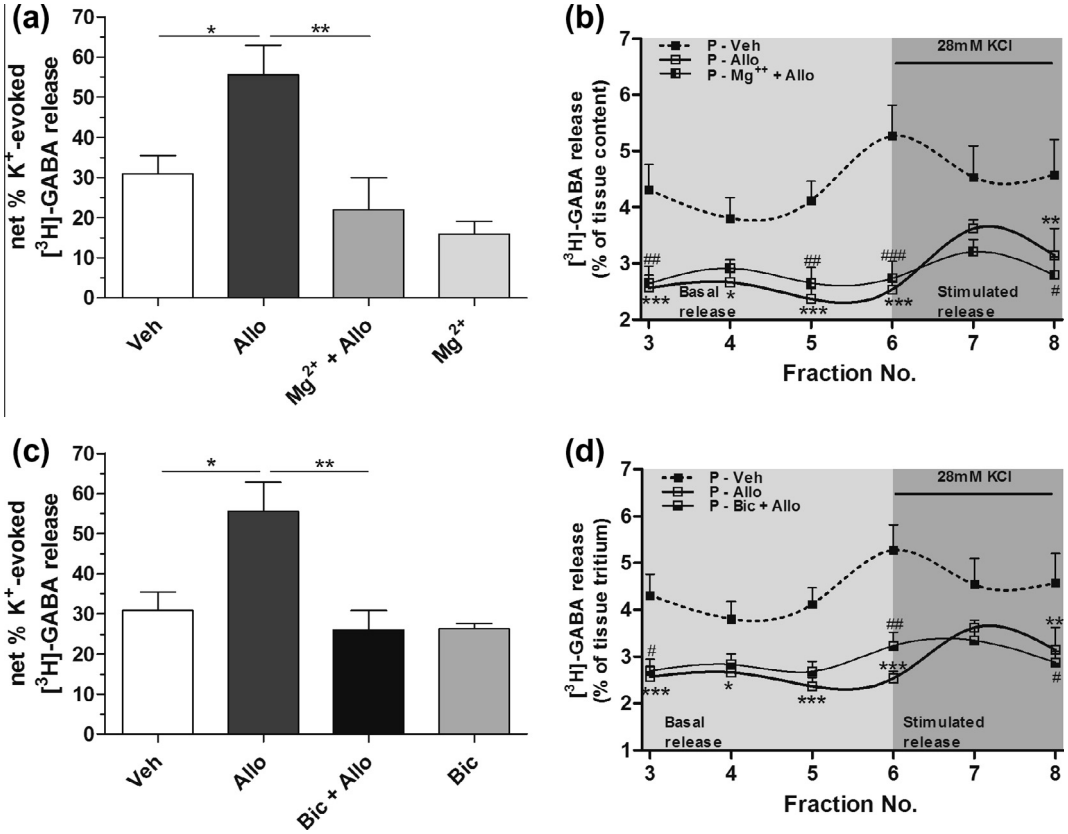

Fig. 6. Blockade of NMDA and antagonism of $GABA_A$ receptors and the effect of allopregnanolone on the [^{3}H]-GABA release in MBH–APOAs of pubertal rats. (a) The blockade of NMDA receptors by the application of KRBG–Mg^{2+} reverts the effect of 120 nM Allo on the net K^+-evoked [^{3}H]-GABA release; (mean ± SEM) $^*p < 0.05$; $^{**}p < 0.01$. (c) The antagonism of $GABA_A$ receptors by the application of Bic reverts the effect of 120 nM Allo (mean ± SEM) $^*p < 0.05$; $^{**}p < 0.01$. The respective profiles of [^{3}H]-GABA release are shown in (b, d); (mean ± SEM) $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Veh vs. Allo); $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ (Veh vs. Mg^{2+} + Allo or vs. Bic + Allo).

Table 1. Averages of % basal [^{3}H]-Glu and [^{3}H]-GABA release. Allopregnanolone increases the basal [^{3}H]-Glu release in MBH–APOAs only in VO rats (VO – Allo vs. PP – Allo; $^{**}p < 0.01$). The application of Mg^{2+} in the superfusion medium reduces the basal release of [^{3}H]-Glu (VO – Mg^{2+} + Allo vs. VO – Allo; $^{\S}p < 0.05$). The basal release of [^{3}H]-GABA is increased in P rats (P – Veh vs. PP – Veh; $^{\#\#\#}p < 0.001$). Allopregnanolone reduces the basal [^{3}H]-GABA release (P – Allo vs. P – Veh; $^{\S\S\S}p < 0.001$). Neither Mg^{2+} nor Bic induces any change on the effect of allopregnanolone on the basal [^{3}H]-GABA release ($^{\S\S}p < 0.01$ vs. P – Veh). Data represent mean ± SEM of the averages of the % [^{3}H]-Glu or [^{3}H]-GABA release among the fractions No. 3, 4 and 5 for each age of development and experimental treatment group

	Veh	Allo	Mg^{2+} + Allo	Bic + Allo
[^{3}H]-Glu				
PP rats	2.84 ± 0.23	2.55 ± 0.22		
VO rats	3.43 ± 0.34	4.51 ± 0.42**	2.98 ± 0.37§	3.42 ± 0.22
P rats	3.68 ± 0.24	3.64 ± 0.35		
[^{3}H]-GABA				
PP rats	1.66 ± 1.11	2.41 ± 1.88		
VO rats	2.60 ± 0.15	2.23 ± 0.17		
P rats	4.12 ± 0.58$^{\#\#\#}$	2.54 ± 0.16§§§	2.59 ± 0.26§§	2.74 ± 0.21§§

of [^{3}H]-Glu in MBH–APOA of VO rats (1-way ANOVA, $p < 0.05$) but the antagonism with Bic did not induce any statistical difference compared to the Veh group (1-way ANOVA; $p > 0.05$) (Table 1).

The 2-way ANOVA of the average basal [^{3}H]-GABA release demonstrated that there was a strong interaction between the two analyzed factors (stage of development and experimental treatment) [$F = 9.93$; DFn = 2; DFd = 35; $p = 0.0004$]. The average basal release increased as rats developed from PP to P stages [$F = 13.99$; DFn = 2; DFd = 35; $p < 0.0001$] being the differences statistically significant between P (Veh-treated) and PP (Veh-treated) rats ($p < 0.001$) (Table 1). On the other hand, according to the 1-way ANOVA, the treatment with allopregnanolone induced a reduction on the average basal release in P rats (Allo vs. Veh; $p < 0.001$) although neither magnesium nor Bic could reverse this effect when they were co-administered with allopregnanolone to the superfusion media (Veh vs. Mg^{2+} + Allo; $p < 0.01$ and Veh vs. Bic + Allo $p < 0.01$) (Table 1).

DISCUSSION

One important aim that we investigated in this work was the hypothalamic expression and activity of 3α-HSOR, the enzyme that catalyzes the conversion of 5α-DHP toward allopregnanolone. The finding that both, the mRNA expression and the enzymatic activity are higher in P than PP rats, suggests the existence of regulatory mechanisms that could function in this stage of the sexual development. The studies on the regulation of this enzyme have been carried out in several species and in different brain regions, and the results are variable. In the rat brain, the mRNA and activity of 3α-HSOR appear to be found almost exclusively in type I astrocytes (Melcangi et al., 1993), however in mice depending on the brain region examined, the enzyme can be also expressed in glutamatergic or GABAergic neurons (Agís-Balboa et al., 2006). There are not enough data regarding the regulation of the enzymatic activity and the gene expression of this enzyme in the rat brain. It has been shown that there is a higher activity of 3α-HSOR before the puberty compared with the adulthood but these studies have been performed in male rats (Eechaute et al., 1999). Recent findings suggest that the activity of key neurosteroidogenic enzymes may be regulated by neurotransmitters that are in turn modulated by neurosteroids, indicating the existence of ultra-short regulatory feedback loops by which the neurosteroids may regulate their own biosynthesis (Do Rego et al., 2009). Alternatively, the reports that describe circadian (Corpéchot et al., 1997) and estrous cycle-dependent fluctuations of the brain allopregnanolone (Genazzani et al., 1995), support the idea of transcriptional hormonal controls of 3α-HSOR. This evidence together with our results suggests that the expression of this enzyme may be regulated by the hormonal changes that occur in puberty.

The increased expression of 3α-HSOR as the sexual maturation progresses could imply a subsequent augment in the biosynthesis of allopregnanolone. This possibility must be further explored since there are not enough data regarding the hypothalamic levels of allopregnanolone through the P development of female rats. However it is important to remark that increased serum levels of allopregnanolone have been detected during the P development in humans (Fadalti et al., 1999) which suggests that the neurosteroid could be important to modulate some neuroendocrine mechanisms related to puberty. A regulated increase in the hypothalamic levels of the neurosteroid during the puberty could explain in part our neurochemical results that indicate a differential *in vitro* reactivity to allopregnanolone of the hypothalamic GABAergic and glutamatergic circuitries during the P development. Such enhanced sensitivity to allopregnanolone may reflect an adaptive mechanism whereby the hypothalamic neuronal networks would become more susceptible to the endogenous variations of the neurosteroid that occur during P development.

It is known that several neurosteroids may influence the release of neurotransmitters (Zheng, 2009). In a previous report, we demonstrated that allopregnanolone at a micromolar concentration stimulated the glutamate release from MBH–APOAs of adult rats through the possible interaction with NMDA receptors (Giuliani et al., 2011). That result led us to ask whether the neurosteroid could also act

during puberty. In this study we tested a 500-fold lower concentration, based on the fact that the neurosteroid at nanomolar concentration appears to allosterically modulate $GABA_A$ receptors instead of directly activating them. Also, with the 120 nM dose we aimed to find results with physiological rather than pharmacological implications. Even though the physiological concentration of allopregnanolone is in general at or below tens of nanomolar (Corpéchot et al., 1993; Cheney et al., 1995) under some conditions (e.g. after stress or pregnancy) the levels can increase to hundreds of nM.

The finding that allopregnanolone under depolarizing (high K^+ concentration) conditions stimulates the release of both neurotransmitters only in P rats (Figs. 3a and 4a), suggests that this neurosteroid is required to modulate the release of key neurotransmitters when puberty is already established instead of at earlier stages of the female sexual development. Taking into account these results, we proposed to determine if these effects were due to modulatory actions of allopregnanolone on the hypothalamic NMDA and $GABA_A$ receptors.

The lack of the stimulatory effect of allopregnanolone on the K^+-evoked glutamate and GABA release when NMDA receptors were blocked with Mg^{2+} (in glutamate and GABA release experiments) or antagonized with AP-7 (in glutamate release experiments) (Figs. 5a and 6a), suggests an effect of the neurosteroid on NMDA receptors and support our previous report in adult rats (Giuliani et al., 2011). This response would be mediated by presynaptic NMDA receptors that regulate glutamate (Aoki et al., 1994) and GABA (Duguid and Smart, 2004) release. It has been shown that the release of many neurotransmitters is regulated by specific presynaptic receptors. In glutamatergic terminals, the existence of NMDA presynaptic receptors that positively control the glutamate release in different areas of central nervous system e.g. the entorhinal cortex (Woodhall et al., 2001; Yang et al., 2008), visual cortex (Aoki et al., 1994; Li et al., 2008), somatosensory cortex (Brasier and Feldman, 2008) and nucleus accumbens has been demonstrated (Huang et al., 2011). Likewise, it has been demonstrated that GABAergic interneurons might have presynaptic NMDA receptors that positively control the GABA release (Duguid and Smart, 2004; Tarasenko et al., 2011; Xue et al., 2011). The present results suggest that allopregnanolone would interact with NMDA receptors. It has been reported that allopregnanolone modulates GABAergic neurotransmission involving an NMDA receptor-mediated mechanism in the central amygdale (Wang et al., 2007) and additionally, we have shown NMDA receptor-mediated effects of allopregnanolone on the striatal dopamine release (Cabrera et al., 2002) and on the hypothalamic glutamate release in adults (Giuliani et al., 2011). Thus, the growing evidence showing the existence of presynaptic NMDA receptors throughout the rat brain, and the possible interaction with allopregnanolone, lead us to propose that in hypothalamic glutamatergic and GABAergic terminals, these presynaptic receptors might be also operating, and that allopregnanolone could modulate their activity to facilitate the respective neurotransmitter release under depolarizing conditions.

Regarding the antagonisms of $GABA_A$ receptors, there was no reversion in the effect of allopregnanolone on the net K^+-evoked glutamate release (Fig. 5c) but there was a reversion in the K^+-evoked GABA release (Fig. 6c) when the antagonist Bic was applied in the superfusion medium. Several studies have demonstrated stimulatory mechanisms of presynaptic $GABA_A$ receptors (Marty and Llano, 2005). For GABA release the existence of presynaptic $GABA_A$ receptors that induce the release of this neurotransmitter in several brain regions including the cerebellum, hippocampus and preoptic area has been demonstated (Haage and Johansson, 1999; Uchida et al., 2002). For glutamate release there are a number of observations that describe $GABA_A$ receptors that activate the release of this neurotransmitter in several regions of the brain such as the hippocampus (Jang et al., 2006), cerebellum (Schmid et al., 1998) and locus coereleus (Hitoshi et al., 2005). As well, it has been reported that the activation of $GABA_A$ receptors induces glutamate release in the preoptic area (Fleischmann et al., 1995). We cannot ignore the possibility of the presence of such $GABA_A$ receptors controlling the glutamate release; in any case, according to our results these putative receptors would not be responsive to allopregnanolone in MBH–APOA of P rats and this fact could be indicating different developmental- and neuronal phenotype-dependent sensitivities of $GABA_A$ receptors to neurosteroids. It has recently described that the isoform $\alpha4\beta\delta$, that has mainly extra- and pery synaptic locations (Wei et al., 2003), appears to be more sensitive to allopregnanolone than the synaptically located $\alpha1\beta2\gamma2$ (Bianchi and Macdonald, 2003; Zheleznova et al., 2008). The response of this isoform increases in response of endogenous (Lovick et al., 2005) or exogenous hormonal changes (Smith et al., 1998; Maguire and Mody, 2007) and it has been demonstrated that hippocampal expression of $\alpha4$ and δ subunits increases at the onset of puberty (Shen et al., 2005). Thus, glutamate and GABA release could be regulated by allopregnanolone through the modulation of $GABA_A$ receptors, the presynaptic δ-containing isoforms being good candidates for such interactions.

While allopregnanolone increases the release of glutamate and GABA under depolarizing conditions, the basal release of both neurotransmitters appears to be regulated differently. In the case of glutamate, the neurosteroid increases the basal release in VO rats (see Fig. 1c and Table 1). This effect appears to be mediated in part by the modulation of NMDA receptors as it is reverted when magnesium is applied to the superfusion medium (see Table 1). Thus, allopregnanolone might be important to control the glutamatergic tone at the onset of puberty. On the other hand, in the case of GABA, allopregnanolone reduces its basal release in P rats (Fig. 2b) in a manner that appears to be independent of allosteric $GABA_A$ or NMDA receptor modulation. Therefore we cannot exclude the possibility that the neurochemical effects of allopregnanolone are produced by other mechanisms in addition to the allosteric

modulation of membrane receptors. It is known that the exocytosis of neurotransmitters is determined by the number of docked vesicles in the active zone of the axonal terminals and by the average of fusion of these vesicles with the plasmatic membrane. This phenomenon is a complex process that requires the opening of Ca^{2+} channels (Catterall, 1999), the activation of intracellular Ca^{2+} sensors (Bennett, 1999), the action of protein kinase C (Stevens and Sullivan, 1998) and changes in the cytoskeleton dynamics. Charalampopoulos et al. (2005) have demonstrated that allopregnanolone and dehydroepiandrosterone sulfate can directly modify the polymerization of actin monomers and affect the secretion of catecholamines. In addition, it has been shown that another neurosteroid, pregnenolone, binds to microtubule-associated proteins and stimulates microtubule assembly (Murakami et al., 2000). Despite that current knowledge is not enough to account for the effect that allopregnanolone has on the spontaneous release of GABA and glutamate, our results suggest that other mechanisms could also be operating in addition to the allosteric modulation of membrane receptors and this is a possibility that deserves further investigation.

Regardless of the mechanisms involved, allopregnanolone could be playing an important physiological role in the hypothalamus of P female rats as a regulator of the release of both key neurotransmitters. Classically, these neurotransmitters have opposite effects into the brain glutamate being the main excitatory amino acid and GABA the main inhibitory one. In MBH–APOA however, they could be having positive effects on the GnRH neurons that have glutamatergic and GABAergic inputs that regulate their neurosecretory activity. The activation of NMDA receptors has been demonstrated as an important stimulus that induces GnRH secretion (Brann and Mahesh, 1991). As well, it has been reported that the GnRH neurons have $GABA_A$ receptors that produce depolarization instead of hiperpolarization when they are activated by the neurotransmitter GABA (DeFazio et al., 2002; Sullivan and Moenter, 2003). Other groups have shown that GABA and glutamate might integrate their effects in multiple temporal (Roberts et al., 2008) or frequency (Liu et al., 2011) frames to facilitate or inhibit the generation of action potentials in GnRH neurons. In this work we show that allopregnanolone has a similar stimulatory effect under depolarizing conditions on the release of both GABA and glutamate in the period of puberty. On the other hand, we also show that the basal release of both neurotransmitters may be differently regulated by the neurosteroid. Noteworthy is the significant reduction in the basal release of GABA when allopregnanolone is administered in the superfusion medium. While the release of GABA under depolarizing conditions appears to increase the release of GABA, this may not be enough to compensate the reducing effect of the neurosteroid on the basal release. In a set of recent experiments conducted in our laboratory we have seen that 120 nM allopregnanolone reduces the GnRH secretion from MBH–APOAs of P rats *in vitro* (unpublished data). Those tissues were incubated with a bath of KRBG buffer supplemented with the neurosteroid under normal potassium conditions. Although the analysis of these changes is beyond the scope of this article, the correlation between those and the present results deserves to be mentioned. Considering that GABA might have stimulatory actions on GnRH and that the above-mentioned experiments of GnRH release were not conducted under depolarizing conditions, the decreased GnRH secretion could be a consequence of the expected reduced basal GABA levels due to the presence of allopregnanolone. Therefore it is tempting to speculate that allopregnanolone at nanomolar concentrations could indirectly modulate the GnRH neuronal functionality through its effects on the activities of the afferent neuronal circuitries but such effects would depend on the integration of specific neurochemical conditions. Our findings strongly support the hypothesis that allopregnanolone would modulate the phenomena of neurosecretion associated with the puberty although the precise conditions that induce stimulatory or inhibitory actions should be further studied.

CONCLUSIONS

In this work we have shown that the enzyme that synthesizes allopregnanolone is more active and expresses highly during puberty. Additionally we have demonstrated that 120 nM allopregnanolone *in vitro* regulates the hypothalamic spontaneous and evoked release of glutamate and GABA in a developmental-dependent manner. We have also shown that these effects could be mediated by the modulation of membrane receptors but also proposed that other cellular mechanism could be involved. Altogether, our results suggest that the modulatory action of allopregnanolone in the hypothalamus could have an important biological function to the accurate 'fine tuning' of the neuronal circuitries that control the neurosecretion related to the sexual development of the female rat. Such effects could increase possibly, the efficacy of other well-known factors such as estradiol, progesterone and kisspeptin on the GnRH network activity.

AUTHOR CONTRIBUTIONS

FAG and CE wrote the paper and analyzed the data; FAG, CE and RC designed the research; FAG, CE, SC and VB performed research; RY, ML and RC supervised the research and the writing of the article.

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