# Adenohypophyseal and hypothalamic GABA B receptor subunits are downregulated by estradiol in adult female rats

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## Abstract

Gamma-aminobutyric acid (GABA) participates in neuroendocrine regulation. Since steroid hormones have been shown to modulate the GABAergic system, here we evaluated the effect of chronic in vivo estradiol administration on GABA B receptor ($GABA_BR$) expression.

$GABA_{B1}$ and $GABA_{B2}$ subunits were analyzed by Western Blot and RT-PCR, in hypothalami and anterior pituitaries of adult female rats: a) treated for 1 week with estradiol-valerate (a single dose of 100 μg /kg: E1), b) implanted with a 10 mg pellet of estradiol-benzoate for 5 weeks (E5) or c) on proestrous (P) d) ovariectomized (OVX). Pituitary $GABA_BR$ levels were correlated to a biological effect: baclofen, a $GABA_BR$ agonist, action on intracellular calcium titers ([Ca2+]i) in pituitary cells.

E5 pituitaries showed a significant decrease in the expression of $GABA_{B1}$ and $GABA_{B2}$ mRNAs compared to P. The $GABA_{B1a}$ splice variant of $GABA_{B1}$ was always more abundant than $GABA_{B1b}$ in this tissue. Similar to the pituitary, hypothalamic $GABA_{B1}$ and $GABA_{B2}$ mRNAs decreased in E5; this was confirmed at the protein level. In the hypothalamus $GABA_{B1b}$ was the main variant expressed in P rats, and was the one significantly sensitive to estradiol-induced decrease, as determined by Western Blots. Castration did not modify $GABA_BR$ expression with regards to P in either tissue. In P pituitary cells baclofen induced a decrease in [Ca2+]i, in contrast this effect was lost in E5 cells.

We conclude that chronic estradiol treatment negatively regulates the expression of the $GABA_BR$ subunits in the pituitary and the hypothalamus. This effect is coupled to a loss of baclofen action on intracellular calcium in pituitary cells.

## Introduction

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS). It also participates in the control of hypophyseal secretion, acting at the CNS and directly at the pituitary level (Demeneix et al., 1986; Donoso and Banzan, 1984; Fiszer de Plazas et al., 1982; Grandison, 1981; Libertun et al., 1979; Tuomisto and Mannisto, 1985). Pituitary GABA derives from tuberoinfundibular GABA, the intermediate pituitary lobe and local synthesis (Gamel-Didelon et al., 2002; Vincent et al., 1982).

This amino acid neurotransmitter acts on three types of receptors, $GABA_A$, $GABA_B$ and $GABA_C$ receptors ($GABA_A$Rs, $GABA_B$Rs and $GABA_C$Rs). $GABA_{A/C}$Rs are ionotropic and belong to the superfamily of ligand-gated ion channels. $GABA_B$Rs are metabotropic, functionally coupled to Gi/0 proteins, specifically activated by baclofen and their "in vitro" activation inhibits PRL and gonadotropins (Lux-Lantos et al., 1992; Rey-Roldán et al., 1996), and stimulates GH secretion (Gamel-Didelon et al., 2002). In addition, they are also expressed in melanotropes, participating in the regulation of αMSH output (Purisai et al., 2005). We have previously described that anterior pituitary $GABA_B$Rs are negatively coupled to voltage-gated calcium channels (VSCC) through Pertussis toxin sensitive G proteins (Lux-Lantos et al., 2001), similar to CNS $GABA_B$Rs (Bowery, 1999).

$GABA_B$Rs are heterodimers formed by a $GABA_{B1}$ and a $GABA_{B2}$ subunits (Bowery et al., 2002; Kaupmann et al., 1998). There are two main isoforms of $GABA_{B1}$, $GABA_{B1a}$ and $GABA_{B1b,}$ generated by distinct promoters and differing in their N-terminal extracellular domain (Kaupmann et al., 1997). Previously, we reported particular pituitary $GABA_B$R ontogenic expression patterns in both sexes in the rat (Bianchi et al., 2001), neonatal testosterone being involved in sexual differences encountered (Bianchi et al., 2004). We have also observed ontogenic sex differences in $GABA_B$R expression in rat hypothalamus (Bianchi et al., 2005). Chronic exposure to estrogen has profound effects on the hypothalamic-pituitary

unit. Among the plethora of effects described for this steroid on the pituitary, the induction of a very significant hyperplasia and hypertrophia of lactotropes, with pituitary enlargement, has been extensively characterized (De Nicola et al., 1978; Gonzalez et al., 2000). Furthermore estrogen treatment produces involution of somatotrophs and gonadotropin-producing cells (De Nicola et al., 1978). In addition, it has been shown that estrogens alter the sensitivity of adenohypophyseal cells to stimulatory and inhibitory factors, such as dopamine, TRH, angiotensin II and GABA (Apud et al., 1985; Diaz-Torga et al., 1998; Livingstone et al., 1998; Suarez et al., 2003; Suarez et al., 2004). Moreover, ovarian steroid hormones modulate GABAergic neurotransmission in various brain areas by altering $GABA_AR$ function or subunit composition (Herbison and Fenelon, 1995; Schumacher et al., 1989; Weiland and Orchinik, 1995). Steroid hormones have also been reported to modify $GABA_BRs$ function or expression in the CNS. Binding of baclofen to neocortical, hippocampal or hypothalamic membranes varied as a function of the estrous cycle, with the lowest binding occurring at different stages of the estrous cycle depending of the area analyzed (al-Dahan et al., 1994). Furthermore, in ovariectomized rats, subcutaneous injection of progesterone alone, without estrogen priming, increased the binding of baclofen to $GABA_BRs$ in the neocortex (al Dahan and Thalmann, 1996). With regard to estrogens, it has been reported that a short exposure to estradiol rapidly blunts the inhibitory response evoked by $GABA_BR$ stimulation in discrete populations of hypothalamic neurons by uncoupling these receptors from $K^+$ channels (Lagrange et al., 1996). It has also been shown that estradiol enhances the Pertussis toxin ADP-ribosylation of Gi/0 proteins in the striatum (Kelly and Wagner, 1999). In addition, in the pituitary, estradiol induces a decrease in the expression of Gi/0 proteins, essential for $GABA_BR$ function (Livingstone et al., 1998).

In this work we evaluated the effect of chronic “in vivo” estradiol exposure on $GABA_BR$ subunits expression in neuroendocrine tissues: the hypothalamus and the anterior pituitary. In

correlation we determined if differences in $GABA_BR$ expression encountered at the pituitary level modified baclofen action on intracellular calcium concentration in pituitary cells.

## Materials and Methods

### Animals

Female Sprague-Dawley rats from the Instituto de Biología y Medicina Experimental colony were used. Adult animals (200-250 g) were implanted with subcutaneous pellets of 10 mg of estradiol-benzoate for a period of five weeks (E5) or injected with a single sc dose of estradiol-valerate (100 μg/kg, Progynon Depot, Schering, Buenos Aires, Argentina, dissolved in castor oil) for studies of one week estradiol treatment (E1). Determinations were also performed in proestrous rats (P) and in ovariectomized animals, sacrificed two weeks postcastration (OVX), both groups injected with castor oil. All groups of animals were sacrificed by decapitation between 9.00 and 10:00 AM, following protocols for animal use approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) and by the NIH.

### Membrane preparation

Anterior pituitaries, separated mechanically from neurointermediate lobes with very fine pincers under a magnifying glass, and hypothalami (limited anteriorly by the optic chiasma, laterally by the hypothalamic fissures, posteriorly by the mammilary bodies and in depth by the subthalamic sulcus, and including the preoptic-suprachiasmatic area) from proestrous and estrogenized female rats were collected and the membrane fraction was isolated, as previously described (Bianchi et al., 2001). Briefly, tissues were homogenized in 10 volumes of ice-cold 0.32 M sucrose, containing 1 mM $MgCl_2$, 1 mM $K_2HPO_4$ with a glass/teflon homogenizer. Membranes were centrifuged at 750 g, the pellet was resuspended and the centrifugation repeated. The supernatants were pooled and centrifuged at 18000 g for 15 min. The pellet was osmotically shocked, centrifuged at 39000 g, resuspended in 50 mM Tris-HCl, 2.5 mM $Cl_2Ca$, pH 7.4 (10 vol/g of original tissue). Membranes were frozen at -70º C.

Western Blot analysis

Western blot analysis of $GABA_BR$ subunits was performed as previously described (Bianchi et al., 2001; Bianchi et al., 2004). Briefly, 50 μg of proteins of hypothalamus or pituitary membrane preparations were subjected to 8 % SDS-PAGE. Proteins were transferred onto nitrocellulose by standard wet electrophoretic transfer in 0.2 M phosphate buffer. Blots were blocked in NETG buffer (159 mM NaCl; 5 mM EDTA; 50 mMTris-HCl, pH 7.4; 0.05 % Triton X-100; 0.25 % gelatin) overnight at 4ºC. $GABA_BR$ subunits were detected by incubating for one hour at room temperature with antibodies Ab174.1 (1:3000) (Malitschek et al., 1998) and AbC22 (1:3000) (Kaupmann et al., 1998) directed against the C-terminal epitopes of $GABA_{B(1a/b)}$ or $GABA_{B2}$ subunits, respectively. Secondary antibody was horseradish peroxidase coupled (1:3000, Santa Cruz Biotechnology, Inc., CA). Blots were washed following each antibody incubation for 50 min with NETG. To ensure comparable protein load, monoclonal antibodies directed against β-actin (1:1000, Santa Cruz Biotechnology) or α-sintaxin (1:3000, Sigma, St Louis MO) were used (Bianchi et al., 2001; Malitschek et al., 1998). Detection was performed using an enhanced chemiluminiscence Western Blot analysis system (Western Blotting Chemiluminescence Luminol Reagent, Santa Cruz Biotechnology). Quantification of immunoblots was performed with Imagequant soft. In the case of α-sintaxin, as previous data showed that estrogen could alter its expression in certain tissues (Brake et al., 2001; Majo et al., 1999), we performed an ANOVA for α-syntaxin levels in the different group studied without observing significant differences ($p=0.5$).

Total RNA preparation and RT-PCR analyses

RNA isolation of adenohypophyses and hypothalami was performed using Trizol RNA isolation reagent (Invitrogen Life Technologies, Buenos Aires, Argentina,). The RNA concentration was determined based on absorbance at 260 nm and its purity was evaluated by

the ratio of absorbancies at 260 nm/280 nm (>1.8). RNA quality was assessed running random samples on denaturated gels. RNAs were kept frozen at -70°C until analyzed.

Semiquantitative RT-PCRs (reverse transcription-polymerase chain reaction) for the detection of the $GABA_{B1}$ and $GABA_{B2}$ subunits mRNAs were performed using the expression of β-actin mRNA as control of the variation in the RNA concentration in RT reaction, as previously described (Bianchi et al., 2004). Briefly, total RNA (0.5 μg) was reverse-transcribed into cDNA using 200 U Moloney murine leukemia virus transcriptase (MMLV, Invitrogen Life Technologies), primed with $oligo(dT)_{15}$ (Biodynamics SRL, Buenos Aires, Argentina). In all RT reactions a negative control, lacking MMLV, was included.

The PCRs for the three transcripts assayed consisted of a 5 min hot start step followed by 28 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min and extension at 72 ºC 1 min, with a final extension for 10 min at 72ºC after the last cycle. PCR amplifications were carried out using Taq DNA polymerase (Invitrogen-Life Technologies, USA) using specific oligonucleotide primers. In these conditions a linear relationship in detection was attained for different amounts of cDNA, obtained from 0.5 μg reversed-transcribed RNA. For pituitary samples, 2 μl of a 1/3 dilution of the RT reaction for $GABA_{B1,}$ 2 μl of a 1/2 dilution of the RT reaction for $GABA_{B2}$ and 2 μl of a 1/250 dilution of the RT reaction for β-actin were used.

When hypothalamic samples were used, 2 μl of a 1/16 dilution of the RT reaction for $GABA_{B1;}$ 2 μl of a 1/4 dilution of the RT reaction for $GABA_{B2}$ and 2 μl of a 1/250 dilution of the RT reaction for β-actin were amplified by PCR. Possible genomic DNA contamination was discarded since primers were designed to encompass exon-intron boundaries and contaminating genomic DNA would have been easily detected. The primers for $GABA_{B1}$ subunit (Bio Synthesis, Lewisville, TX), that did not distinguish between the splice variants $GABA_{B1a}$ and $GABA_{B1b}$, were synthesized based on the sequences described by Schuler et al

(Schuler et al., 2001): *forward*: 5´-AGCTGACCAGACCTTGGTCAT-3´; *reverse*: 5´-AACTGGCTTCTCCCTATGTGG-3´, amplifying a theoretical fragment of 254 bp from mRNA and of 690 bp from genomic DNA.

The primers to detect $GABA_{B2}$ mRNA (Bio Synthesis, Lewisville, TX) were synthesized based on the sequences published by Mayerhofer et al (Mayerhofer et al., 2001): *forward*: 5´-CATCATCTTCTGCAGCAC-3´; *reverse*: 5´-TCTGTGAAGTTGCCCAAG-3´ amplifying a fragment of 354 bp from mRNA. With the conditions used no product was obtained from genomic DNA.

The primers for β-actin (Invitrogen Life Technologies) were synthesized following a published sequence (Brussaard et al., 1999): forward: 5´-GGAAATCGTGCGTGACAT-3´; *reverse*: 5´-GGAAGGTGGACAGTGAGG-3´, amplifying a fragment of 440 bp from mRNA and 652 bp for genomic DNA. To ensure accuracy of the PCR, samples were run without template (control). The reactions were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The gels were exposed to UV with a white/ UV ultraviolet transilluminator (UVP laboratory Products) and the images quantified using the Scion Images NIH software.

Anterior pituitary cells

Anterior pituitaries cells from five week estradiol-treated rats or from proestrous rats were obtained as previously described (Lux-Lantos et al., 1992; Rey-Roldán et al., 1996). Briefly, adenohypophyses were rapidly removed and placed in freshly prepared Krebs-Ringer bicarbonate buffer without $Ca^{2+}$ and $Mg^{2+}$. Pituitaries were cut into small pieces and incubated in 0.2% trypsin for 30 min. After addition of DNase and limabean trypsin inhibitor, cells were dissociated by gentle trituration of the fragments and filtered through Nytex.

Intracellular $Ca^{2+}$ measurements

Intracellular $Ca^{2+}$ was performed as previously described using Fura-2/AM (tetracetoxymethylesther-Fura 2) as a fluorescent indicator (Lux-Lantos et al., 2001; Suarez et al., 2004). Briefly, adenohypophyseal cells were incubated for 30 min in a buffered saline solution (BSS: 140mM NaCl, 3.9mM KCl, 0.7mM $KH_2PO_4$, 0.5mM $Na_2HPO_4.12H_2O$, 1mM $CaCl_2$, 0.5mM $MgCl_2$, 20mM Hepes, pH 7.5) in the presence of FURA-2/AM 1.5μM, 10mM glucose, 0.1% BSA. Cells were then washed in BSS without FURA-2/AM. The cells were suspended in a density of 1.5 to $2*10^6$cells/ml BSS. Fluorescence was measured in a spectrofluorometer with continuous stirring, thermostat adjusted to 37ºC, and injection chamber (Jasco Corporation, Tokyo). Drugs were injected (5μl) into the chamber as a 100-fold concentrated solution without interruption of recording. Intracellular $Ca^{2+}$ levels were registered every second by exposure to alternating 340 and 380 nm light beams and the intensity of light emission at 505 nm was measured. In this way light intensities and their ratio (F340/F380) were followed. The preparation was calibrated determining maximal fluorescence induced by 0.1% Triton X-100 (Fmax) and minimal fluorescence (Fmin) in the presence of 6mM EGTA (pH adjusted to over 8.3). Values were corrected for dye leakage and auto fluorescence. Experiments were repeated 7 times. Intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated according to Grynkiewicz et al. (Grynkiewicz et al., 1985).

Results in $[Ca^{2+}]_i$ were expressed as the percent variation with respect to basal levels. Average levels (in nM) measured 30 sec before buffer or baclofen addition (min 3) were considered as basal levels. To quantify differences between treatments and groups, average levels between min 3 and 4.5 (stimulus applied at min 3) were calculated for each experiment.

Drugs

Unless specified, all chemicals were purchased from Sigma, St. Louis, MO. L-baclofen was a gift from Novartis, Buenos Aires.

Trypsin was purchased in Gibco, Buenos Aires, Argentina; DNase was from Worthington Biochemical Corp., Lakewood, NJ.

Statistical Analysis

Differences in Calcium levels were analyzed by two-way analysis of variance followed by Newmans-Keuls`test. Differences in $GABA_BRs$ subunit expression by Western Blots and mRNA expression by RT-PCR were analyzed by either one-way or two-way analysis of variance followed by Newmans-Keuls`test or Tuckey HSD for unequal N.

## Results

Effect of estradiol on pituitary $GABA_B$ receptor subunit expression

To evaluate the effect of chronic estradiol treatment on $GABA_BR$ expression, receptor subunits were determined, both at the protein and mRNA levels, in pituitary and hypothalamic membranes from rats treated for 1 or 5 weeks with the steroid and from proestrous animals.

The level of expression of the $GABA_{B1a}$ and $GABA_{B1b}$ variants of the $GABA_{B1}$subunit in anterior pituitary gland membranes from 1 or 5 week estradiol-treated rats was quite low and did not differ from the expression in membranes from proestrous animals. In all cases $GABA_{B1a}$ was significantly more abundant than $GABA_{B1b}$ (Fig. 1).

$GABA_{B2}$ subunit protein expression was barely detectable in pituitary membranes from estradiol treated rats (not shown), similar to previous results during ontogeny and in adulthood in this gland (Bianchi et al., 2001).

When anterior pituitary mRNA levels were analyzed by RT-PCR, single bands of 254 bp and 354 bp corresponding to the $GABA_{B1}$ and the $GABA_{B2}$ subunits respectively were detected. Five weeks of estradiol treatment induced significant decreases in $GABA_{B1}$ (Fig. 2, upper panel) and $GABA_{B2}$ (Fig. 2, lower panel) expression when compared to proestrous titers.

In pituitaries from castrated females the $GABA_{B1}$ mRNA expression levels were similar to those found in proestrous animals [$GABA_{B1}$/ β actin (AU): OVX: 139.9 ± 6.0 (n=4) vs. P: 128.7 ± 7.8 (n=6), ns].

Effect of estradiol on hypothalamic $GABA_B$ receptor subunit expression

In contrast to the pituitary, $GABA_{B1b}$ was the most abundant splice variant of the $GABA_{B1}$ subunit in the hypothalamus of adult proestrous rats, as revealed by Western Blots. This hypothalamic pattern of expression was conserved after one week of estradiol treatment, but was lost after five weeks of treatment (Fig. 3, upper panel). This occurred because a

significant decrease in the $GABA_{B1b}$ protein was observed after 5 weeks estradiol treatment, while $GABA_{B1a}$ showed a small decrease which did not attain statistical significance (two-way ANOVA: interaction: $p<0.006$). Furthermore, the expression of the $GABA_{B2}$ subunit was also significantly lower in hypothalami from estradiol-treated rats than in proestrous hypothalamic membranes, both after 1 and 5 weeks of estradiol treatment (Fig. 3, lower panel).

We next investigated whether hypothalamic $GABA_{B1}$ mRNA was also affected by estradiol treatment. A significant decrease of $GABA_{B1}$ mRNA expression was observed in hypothalami of 5 week estradiol-treated rats when compared to proestrous controls (Fig. 4, upper panel); these levels were not altered after one week of treatment. In addition, hypothalamic $GABA_{B2}$ mRNA expression levels fell after 1 and 5 weeks of estradiol treatment (Fig. 4, lower panel), coinciding with Western Blot results.

Similar to what was observed in the hypophysis, in the hypothalami of ovariectomized rats expression of $GABA_B$ subunits mRNAs did not differ from proestrus [$GABA_{B1}$ mRNA/ β actin mRNA: OVX: 75.26 ± 2.95 (n=4) vs P: 83.23 ± 6.41 (n=5), ns; $GABA_{B2}$ mRNA/ β actin mRNA: OVX: 136.8 ± 15.6 (n=5) vs P: 172.2 ± 4.3 (n=4), ns].

Effect of Baclofen on calcium mobilization in anterior pituitary cells

As a significant decrease in the expression of $GABA_B$Rs was determined after 5 weeks of estradiol treatment in pituitary and hypothalamic membranes, we evaluated if such decrease modified receptor function. For this purpose, the dynamics of intracellular calcium levels in response to baclofen were measured in control adenohypophyeal cells from proestrous rats and in cells from 5 week-estrogenized animals.

In anterior pituitary cells obtained from estradiol-treated animals, baclofen ($5.10^{-4}$M) did not modify basal intracellular calcium, in contrast to its effect on adenohypophyseal cells from proestrous rats, where a significant decrease was observed ($p=0.003$, Fig. 5).

## Discussion

We have previously studied the hormonal effects, mechanism of action and ontogenic expression patterns of anterior pituitary $GABA_BRs$ (Bianchi et al., 2001; Lux-Lantos et al., 2001; Lux-Lantos et al., 1992; Rey-Roldán et al., 1996). In addition, we demonstrated that elevated perinatal testosterone decreased $GABA_{B1}$ subunit expression in the pituitary of infantile male rats and this could also be achieved in females by neonatal testosterone administration (Bianchi et al., 2004). In the hypothalamus, a sexually dimorphic expression of the $GABA_{B1a}$ subunit was also observed on the day of birth, being more abundant in females than in males (Bianchi et al., 2005). Ovarian steroids have been proposed to participate in the regulation of the function and/or expression of $GABA_BRs$ in the CNS (al Dahan and Thalmann, 1996; al-Dahan et al., 1994; Kelly et al., 2002; Kelly and Wagner, 1999; Lagrange et al., 1996; Livingstone et al., 1998; Malyala et al., 2005). To further elucidate estrogen action on $GABA_BRs$, we investigated the effect of chronic estradiol exposure on hypothalamic and pituitary $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ subunits expression and on receptor function in adult female rats.

Estradiol treatment markedly affected $GABA_BR$ expression in the hypothalamic-pituitary unit. In the hypophysis decreases of 33% in $GABA_{B1}$mRNA and 34% in $GABA_{B2}$mRNA were detected after 5 weeks of estradiol exposure in adult female rats. This could not be confirmed at the protein levels, probably due to low levels of expression of this receptor in the gland in comparison to the CNS as proposed by Anderson and Mitchell (Anderson and Mitchell, 1986), and/or to the sensitivity of the method. It has been postulated that estradiol may induce changes in the subunit composition of the $GABA_ARs$ (Herbison and Fenelon, 1995). This does not seem to occur in the case of pituitary $GABA_BRs$, as both proestrous and estrogenized animals showed similar proportion of $GABA_{B1}$ variants, being $GABA_{B1a}$ always more abundant, as we had previously described during ontogeny and in adult male and female

rats (Bianchi et al., 2001; Bianchi et al., 2004). Regarding the effect of estradiol on GABA receptor subunit composition, previous studies have shown that a single estradiol dose induced the disappearance of the low affinity population of $GABA_A$Rs in the pituitary, while two months chronic treatment with the steroid induced the disappearance of the high affinity population of $GABA_A$Rs in the gland (Apud et al., 1985). In addition, concerning the effect of steroid hormones specifically on $GABA_B$Rs, we have recently demonstrated a clear sexually dimorphic pattern of expression in the pituitary as both variants of the $GABA_{B1}$ subunit are more abundant in female than in male infantile rats (Bianchi et al., 2001). This sex difference is the consequence of elevated perinatal testosterone in males; a decrease in $GABA_{B1}$ expression could also be achieved in females by neonatal testosterone administration (Bianchi et al., 2004). The effect of estradiol on adult female pituitary $GABA_B$R expression demonstrated herein together with the previous data point to a general negative regulatory role of these steroid hormones on the expression of pituitary $GABA_B$Rs. This effect may not be specific for $GABA_B$Rs, as $GABA_A$Rs also seem to be affected by this treatment (Apud et al., 1985). In addition, $GABA_B$ receptors are expressed in different populations of anterior pituitary cells and long-term estradiol treatment induces hyperplasia of the gland mostly at the expense of lactotropes (Gonzalez et al., 2000). In our model we cannot determine if the reduction in $GABA_B$ receptors is particular of one cell type or if it is a general phenomenon. Future double immunocytochemistries for receptor presence and cell type will clarify this point.

In the hypothalamus a consistent inhibitory effect of estradiol on $GABA_B$R subunit expression was observed both at the mRNA and protein levels. One week of estradiol treatment was sufficient to induce a 35 % decrease in the expression of $GABA_{B2}$ protein and a 30% decrease in the mRNA levels of this subunit with respect to proestrous levels. No further effect was observed after five weeks of estrogen administration (44 % decrease in the expression of

$GABA_{B2}$ protein and 23 % decrease in the expression of its mRNA). Five weeks of estradiol treatment were necessary to down-regulate the $GABA_{B1}$ subunit. While mRNA levels, which do not discriminate between the $GABA_{B1a}$ and $GABA_{B1b}$ isoforms, decreased 33%, we determined by Western Blot analysis a significant 65% decrease in the expression the $GABA_{B1b}$ and a 42% decrease in $GABA_{B1a}$ proteins, this last effect not attaining significance. In these animals hypothalamic $GABA_{B1b}$ levels were no longer different from $GABA_{B1a}$ and therefore the characteristic hypothalamic expression pattern of adulthood, with a predominance of the $GABAB_{1b}$ isoform, was lost (Bianchi et al., 2005). This adult-like pattern has also been demonstrated in other brain regions (Fritschy et al., 2004; Malitschek et al., 1998). These results suggest that $GABA_{B1b}$ is the main splice variant sensitive to estrogen regulation, whereas testosterone downregulates both splice variants to a similar degree in infantile rats (Bianchi et al., 2004). Differential regulation of the expression of the $GABA_{B1a}$ and $GABA_{B1b}$ isoforms in neonatal rat brain has already been proposed by Fritschy et al. (Fritschy et al., 2004). Regarding steroid hormone modulation of $GABA_BR$ expression in the CNS, progesterone, in the absence of estrogen priming, has been shown to increase $GABA_BR$ binding in the neocortex (al Dahan and Thalmann, 1996) and this event was related to $GABA_BR$ fluctuation during the estrous cycle (al Dahan et al., 1994). Similar to our observations in total hypothalamus, Francois-Bellan et al. described that chronic estradiol treatment decreased the density of $GABA_BRs$ in the suprachiasmatic nucleus and the striatum, but not in the medial preoptic area, while it failed to significantly affect $GABA_ARs$ (Francois-Bellan et al., 1989). None of these early works differentiated between subunits and/or splice variants of the $GABA_BR$. In addition, our results show that castration does not significantly modify $GABA_BR$ expression in the hypothalamus or the pituitary with regard to levels of proestrus, implying that preovulatory estrogen titers are not sufficient to induce downregulation of these receptors, as is also the case of one week of estradiol treatment for

the $GABA_{B1}$ subunit. Interestingly, androgenization with low testosterone levels (1 μg testosterone propionate/day on days 1 to 4 of life) was also ineffective in downregulating pituitary $GABA_B$Rs in neonatal female rats (Bianchi et al., 2004), suggesting that persistent elevated concentrations of the steroids are necessary to alter $GABA_B$R expression.

Estradiol has also been shown to alter $GABA_B$R function, as short term estradiol exposure uncouples $GABA_B$Rs from G protein-gated inwardly rectifying $K^+$ (GIRK) channels in certain populations of hypothalamic neurons (Lagrange et al., 1996; Malyala et al., 2005). An estradiol-induced downregulation of the expression of Gi/0 proteins, indispensable for $GABA_B$ signaling, has also been reported (Kelly and Wagner, 1999; Livingstone et al., 1998).

Regarding receptor function, we have previously described that $GABA_B$R activation by baclofen induced a transient fall in basal intracellular calcium levels in proestrous adenohypophyseal cells, without affecting the response to calcium-elevating stimuli like TRH (Lux-Lantos et al., 2001). To evaluate if the estradiol-induced decrease of $GABA_B$Rs modified this response, the dynamics of intracellular calcium levels after baclofen administration were measured in control adenohypophyseal cells from proestrous rats and in cells from 5 week-estrogenized animals. As expected, baclofen was no longer effective in pituitary cells from estrogenized rats. This lack of response is probably due to estradiol-induced downregulation of $GABA_B$Rs, though a desensitizing effect of the steroid cannot de discarded as previous reports have demonstrated alterations in calcium homeostasis in pituitary cells from estrogen-treated animals, including a decreased response to L-type VSCC inhibitors (Gonzalez Iglesias et al., 1999), as well as uncoupling of receptors from Gi/0 proteins (Kelly and Wagner, 1999; Livingstone et al., 1998), all factors involved in the $GABA_B$R signaling pathway.

## Conclusion

For the first time the effect of chronic estradiol exposure on the expression of the $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ subunits in the anterior pituitary and the hypothalamus was evaluated. Our results demonstrate that persistent estradiol has a negative regulatory role on the expression of $GABA_B$Rs in these tissues. The $GABA_{B2}$ subunit is more sensitive to estradiol action than the $GABA_{B1}$ subunit, and the $GABA_{B1b}$ isoform is more sensitive than the $GABA_{B1a}$ variant. On the other hand, castration does not modify $GABA_B$R expression when compared to proestrous levels. The downregulation of pituitary $GABA_B$Rs in estrogenized animals is functionally coupled to a decrease in baclofen action on intracellular calcium levels in these cells.

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## Figure Legends

**Figure 1:** Anterior pituitary $GABA_{B1}$ subunit expression in estradiol-treated rats and proestrous animals determined by Western blot.

Inset: Representative Western blot for $GABA_{B1a}$ (120 kDa) and $GABA_{B1b}$ (100 kDa) isoforms of the $GABA_{B1}$ subunit and β-actin expression. For this and the following figures: KM, molecular weight markers; P: proestrous rats; E1, estradiol treatment for 1 week; E5, estradiol treatment for 5 weeks; Ctx, cortex: positive control.

Results in arbitrary units (AU) are the mean ± SE of 4-5 independent samples and are expressed as the ratio of each $GABA_{B1}$ isoform to β-actin expression. Data were analyzed by two-way ANOVA: interaction: ns, indicating that both subunits follow the same expression pattern among groups; factor subunit: $p<0.001$, a: indicating that $GABA_{B1a}$ levels are higher than $GABA_{B1b}$ levels.

**Figure 2**: Anterior pituitary $GABA_B$ subunits mRNA expression in estradiol-treated rats and proestrous animals determined by RT-PCR.

Insets: representative RT-PCR gels showing the bands for $GABA_{B1}$ (254 bp, upper panel) or $GABA_{B2}$ (354 bp, lower panel) subunits and β-actin expression (440 bp).

Results in arbitrary units (AU) are the mean ± SE of 4-5 independent samples and are expressed as the ratio of the $GABA_B$ subunit to β-actin expression.

Upper panel: $GABA_{B1}$subunit: one-way ANOVA: $p<0.01$, *: significantly different from P and E1 levels.

Lower panel: $GABA_{B2}$ subunit: one-way ANOVA: $p<0.03$, *: significantly different from P.

**Figure 3**: Hypothalamic $GABA_B$ subunits expression in estradiol-treated rats and proestrous animals determined by Western blot.

Insets: Representative Western blots for $GABA_{B1a}$ and $GABA_{B1b}$ isoforms of the $GABA_{B1}$ subunit (120 kDa and 100 kDa, respectively, upper panel) and for GABA B2 (110 kDa, lower panel) and their respective α-syntaxins. Results in arbitrary units (AU) are the mean ± SE of 4-5 independent samples and are expressed as the ratio of each $GABA_{B\ 1a/b}$ isoform (upper panel) or $GABA_{B2}$ subunit (lower panel) to α-syntaxin expression.

Upper panel: $GABA_{B1}$subunit: data analyzed by two-way ANOVA: interaction: $p<0.01$, a: $p<0.01$, significantly different from $GABA_{B1a}$; *: $p<0.01$, significantly different from $GABA_{B1b}$ in P and E1.

Lower panel: $GABA_{B2}$subunit: data analyzed by one-way ANOVA: $p<0.01$, *: significantly different from P.

**Figure 4**: Hypothalamic $GABA_B$ subunit mRNA expression in estradiol-treated and proestrous rats determined by RT-PCR.

Insets: representative RT-PCR gels for $GABA_{B1}$ subunit (upper panel) or $GABA_{B2}$ subunit (lower panel) and their respective β-actins.

Results in arbitrary units (AU) are the mean ± SE of 4-5 independent samples and are expressed as the ratio of the $GABA_B$ subunit to β-actin expression.

Upper panel: $GABA_{B1}$ subunit: one-way ANOVA: $p<0.005$, *: significantly different from proestrous $p<0.01$ and E1 $p<0.05$.

Lower panel: $GABA_{B2}$ subunit: one-way ANOVA: $p<0.01$, *: significantly different from proestrous.

**Figure 5**: Upper panel: representation of % variation of intracellular calcium concentrations ($[Ca^{2+}]_i$) induced by baclofen ($5.10^{-4}$ M, added at min 3) or buffer in anterior pituitary cells from E5 (upper right panel) and proestrous rats (upper left panel). n=7

Lower panel: Average % $[Ca^{2+}]_i$ levels between min 3 and 4.5 (stimulus applied at min 3) from proestrous and E5 cells from 7 independent experiments. Data were analyzed by two-way ANOVA, interaction: $p<0.04$, *: significantly different from buffer in proestrous cells, $p<0.01$.