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Research Report

Expression of gamma-aminobutyric acid B receptor subunits in hypothalamus of male and female developing rats

María S. Bianchi[a], Victoria A. Lux-Lantos[a], Bernhard Bettler[c], Carlos Libertun[a,b,*]

[a]Instituto de Biología y Medicina Experimental-CONICET, V. de Obligado 2490, (C1428ADN) Buenos Aires, Argentina
[b]Faculty of Medicine, Universidad de Buenos Aires, Buenos Aires, Argentina
[c]Department of Clinical-Biological Sciences, University of Basel, Basel, Switzerland

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Abstract

GABA and its receptors show particular ontogenic distributions in different rat brain areas. Recently, $GABA_B$ receptors (GBR) have been described to assemble as heterodimers formed by a GBR1a/b and a GBR2 subunit. Here, the ontogeny of rat GBRs and the pattern of subunit expression in both sexes were determined in the hypothalamus, a critical area for homeostatic regulation. Male and female rats were sacrificed at 1, 4, 12, 20, 28, 38 days of life and at adulthood and hypothalami were removed and frozen. Western blots analysis for GBR1 and GBR2 subunits showed that both were expressed in male and female hypothalamic membranes from day 1 to adulthood. In females, both GBR1a and GBR1b were maximally expressed in newborns and decreased towards adulthood. At birth, expression of GBR1a was significantly higher than GBR1b, while at 38 days, GBR1b was more abundant. In males, GBR1a and GBR1b expression was higher in young animals and decreased gradually showing adult levels between the second and third weeks of age without differences between isoforms. Comparing GBR1 variants levels in hypothalamus between sexes, GBR1a was significantly more abundant in females at birth while at 38 days its expression was higher in males; GBR1b showed no sex differences along development. GBR2 was detected in hypothalami of females and males at all ages; maximum levels were observed at 12 days and adult levels were attained at 38 days, without sex differences. This is the first report on the ontogeny of hypothalamic $GABA_B$ receptors in male and female rats, with a particular developmental pattern of subunit and isoform expression and presenting some sex differences.

Theme: Neurotransmitters, modulators, transporters and receptors
Topic: GABA receptors

Keywords: $GABA_B$ receptor; Hypothalamus; Ontogeny; Rat; Male; Female

1. Introduction

Multiple roles, as metabolite, neurotransmitter and neuro-differentiative agent, have been attributed to gamma-amino-butyric acid (GABA) [39,40]. The early activation of GABA A and GABA B receptors appears to account for the regulation of proliferation, migration and differentiation during central nervous system (CNS) development, prior to their role in neurotransmission [2,10,31]. In addition, GABA may play a decisive role in determining sexual differentiation of critical brain areas, such as the hypothalamus [28]. Moreover, GABA and its receptors have particular ontogenic distributions in different rat brain areas. In this way, many different types of GABA receptors (GBRs) have prominent expression in the embryonic and/or postnatal brain, whereas

Abbreviations: GABA, gamma-aminobutyric acid; GBR, gamma-aminobutyric acid B receptor

* Corresponding author. Instituto de Biología y Medicina Experimental-CONICET, V. de Obligado 2490, (C1428ADN) Buenos Aires, Argentina. Fax: +54 11 4786 2564.
E-mail address: libertun@dna.uba.ar (C. Libertun).

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others are mainly present in the adult brain. This change in subunit composition is essential for normal development in particular brain regions [22]. For example, in rat CNS, the expression of the $GABA_A$ receptor α1 subunit is low at birth, but increases during the first postnatal week, whereas the α2 subunit decreases progressively [12]. Regarding $GABA_B$ binding sites, some authors have described their presence in rodent brain at early stages of life, peaking at regionally specific times during the first 3 weeks of life and then declining to adult levels [7,8,38]; others have described that they either decrease or do not change along ontogeny, depending on the region of the nervous system analyzed [25]. In some cases, changes in the relative expression of receptor splice variants have also been reported [12–14,25].

GABA is a dominant neurotransmitter in the hypothalamus, accounting for almost 50% of all synaptic contacts [11]. This hypothalamic GABAergic system is critical in homeostasis regulation, including significant participation in the control of pituitary function. It regulates vital functions such as the response to stress [20] or reproduction [35]. In this regard, GABA is involved in the regulation of the migration of GnRH neurons from the olfactory placode into the forebrain and subsequently into the hypothalamus [36] and, later on in development, in the regulation of GnRH secretion [16], playing a key role in pubertal eclosion [15,35]; in these events, the participation of GABA A and/or B receptors has been proposed. In addition, distinct patterns of pituitary hormones responses to GABA, through both $GABA_A$ and $GABA_B$ receptor stimulation, have been demonstrated in developing and adult male and female rats [24,29,33,34].

Recently, GBRs have been described to assemble as heterodimers formed by a GBR1 and a GBR2 subunit [4,18,19,21,41]. Initially, two major isoforms of GBR1, GBR1a and GBR1b, arising from differential promoter usage of the GBR1 gene were isolated [18], coding for proteins of 120 kDa and 100 kDa, respectively. Additional splice variants of GBR1 have since been identified [17,32,37], though their physiological importance has not been elucidated. We have recently described the ontogenic pattern of GBR subunits expression in the adenohypophysis, revealing marked sex differences [5], which are determined by prenatal androgen exposure [6].

Taking into consideration the importance of GABA in hypothalamus and the lack of information on developmental hypothalamic GBR expression, the present study was undertaken to determine the ontogeny of GBRs in this area from birth to adulthood and the pattern of subunit expression in both sexes in the rat.

2. Materials and methods

Male and female virgin Sprague–Dawley rats (200–250 g) from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room, with lights on from 0700 to 1900 h. They were given free access to laboratory chow and tap water. All studies were performed according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) and by the NIH. Female rats were mated on proestrus. Pregnant females were kept in individual cages and, after delivery, mothers with their pups were left undisturbed until the day of the experiment. Day of birth was considered as day 1. Ages studied were 1, 4, 12, 20, 28, 38 days of life and adults (2–3 months old). Adult female rats were used in proestrus or at 15 days of lactation. Animals were decapitated around 9:00 AM, to avoid circadian variations. The brains were rapidly removed and placed on ice for dissection. An area limited anteriorly by the optic chiasma, laterally by the hypothalamic fissures, posteriorly by the mammilary bodies and in depth by the subthalamic sulcus was excised. This section included the whole hypothalamus and the preoptic suprachisamatic area [1].

The membrane fraction of the collected hypothalami was isolated as previously described [30]. Briefly, hypothalami were homogenized in 10 volumes of ice-cold 0.32 M sucrose, containing 1 mM $MgCl_2$ and 1 mM K_2HPO_4, pH 7.4, with a glass/teflon homogenizer (pool of two hypothalami for 1, 4 and 12 day-old animals, single hypothalamus for the other groups). Membranes were centrifuged at 750 × *g*, 10 min at 4 °C; the pellet was resuspended and the centrifugation repeated. The supernatants were pooled and centrifuged at 18,000 × *g* for 15 min. The pellet was osmotically shocked, centrifuged at 39,000 × *g*, 20 min, resuspended in 50 mM Tris–HCl; 2.5 mM CaCl2, pH 7.4 (10 volumes/g of original tissue) and washed twice. Membranes were frozen at −70 °C. Number of animals/samples per group: females: day 1: 16 animals = 8 samples, day 4: 16 animals = 8 samples, day 12: 12 animals = 6 samples, day 20: 7 animals/samples, day 28: 6 animals/samples, day 38: 6 animals/samples, adult proestrous rats: 6 animals/samples, lactating rats: 4 animals/samples; males: day 1: 20 animals = 10 samples, day 4: 16 animals =8 samples, day 12: 16 animals = 8 samples, day 20: 8 animals/samples, day 28: 8 animals/samples, day 38: 8 animals/samples, adult rats: 6 animals/samples.

Western blot analysis of GBRs subunits was performed as previously described [5,6]. Briefly, the system was calibrated for protein loading and aliquots of 40 μg of hypothalamic membrane preparations were selected because they gave a good signal without saturation images. After loading, gels were subjected to 8% SDS-PAGE. Proteins were transferred onto nitrocellulose by standard wet elctrophoretic transfer in a 0.2 M phosphate buffer, pH 7.7. Blots were blocked in NETG buffer (150 mM NaCl; 5 mM EDTA; 50 mM Tris–HCl, pH 7.4; 0.05% triton X-100; 0.25% gelatin) for 30 min with three changes of buffer. GBR subunits were detected by incubating for 45 min at room temperature with antibodies generated by Dr. B. Bettler et al.: Ab174.1 (1:3000) which detects GBR1a (120 kDa) and GBR1b (100 kDa) [25] or AbC22 (1:3000) which detects GBR2 (110 kDa) [19]. In all immunoblotting experi-

ments, a monoclonal antibody directed at α-syntaxin (1:3000) was used to normalize protein levels in every lane, as previously described [5,25]. Secondary antibody was peroxidase coupled (1:3000). Blots were washed following antibody incubation for 30 min with NETG. Detection of the antibody was performed using enhanced chemiluminescence Western blot analysis system (Santa Cruz Biotechnology, Inc., Santa Cruz CA). After preliminary testing of different times of development, 2.5 min were selected to avoid maximum saturation of the signals and this time was kept standard for all the gels. To optimize comparison between sexes and among ages, at least one sample of each sex and age was run in the same gel and to prevent false variability between gels the same cerebral cortex standard was used in all of them. Quantification of immunoblots was performed with Imagequant soft analyzing bands for volume.

Statistical analysis: ontogenic and sexual differences in GBR subunit expression were analyzed by two-way analysis of variance followed by Tuckey HSD for unequal N test (Statistica V5). $P < 0.05$ was considered significant.

3. Results

Both GBR1 splice variants, GBR1a and GBR1b, were expressed in developing female and male hypothalami from day 1 of life to adulthood. In females (Fig. 1), GBR1a was maximally expressed on the first day of life and significantly decreased towards adulthood, attaining the lowest levels at 38 days; adult levels were similar to 28 day-old animals; GBR1b also decreased towards adulthood, though less markedly, attaining adult levels at 20 days. When evaluating relative GBR1 isoforms expression levels during development in females, GBR1a was significantly more abundant than GBR1b in 1 day-old animals while the inverse was observed in 38 day-old female hypothalamic membranes, where GBR1b was more abundant ($P < 0.05$). This inversion in isoform abundance was also detected in adult females, though not attaining statistical significance ($P < 0.07$). GBR1 levels in lactating rats were similar to proestrous animals [GBR1a/syntaxin (AU): proestrous: 2.82 ± 0.32 ($n = 6$) vs. lactating: 2.50 ± 0.10 ($n = 4$), ns; GBR1b/syntaxin (AU): proestrous: 4.29 ± 0.47 ($n = 6$) vs. lactating: 3.74 ± 0.27 ($n = 4$), ns].

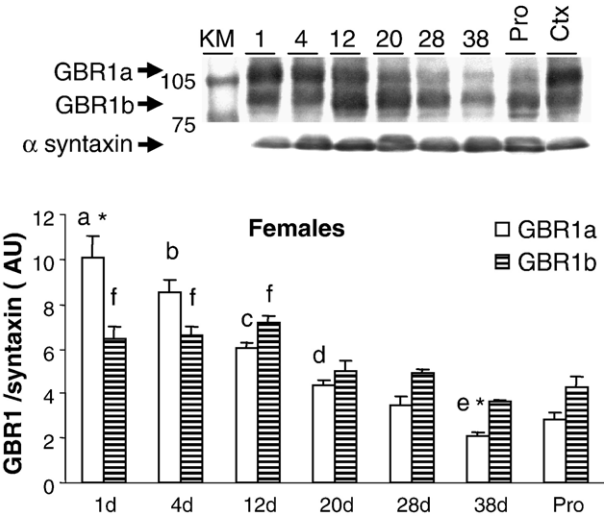

Fig. 1. GBR1 subunit expression in female hypothalami during development. Upper panel: representative Western blot of GBR1a/b isoforms: ages 1, 4, 12, 20, 28 and 38 days and adult proestrous rats (Pro). For this and next figures: KM: molecular weight markers; Ctx: cortex positive control; α-syntaxin (lower part of each blot, apparent molecular mass < 45 kDa) is an internal control of protein loading. Lower panel: results in arbitrary units (AU) are the mean ± SE of 5–8 independent samples and are expressed as the relation of each subunit with regard to its α-syntaxin control. Two-way ANOVA taking as factors GBR1 isoforms and age: interaction: $P < 0.001$, a: GBR1a different from 12 days onwards, b: GBR1a different from 20 days onwards, c: GBR1a different from 28 days onwards, d: GBR1a different from 38 days and adult levels, e: GBR1a different from all other ages, f: GBR1b different from 38 days and adult, *: GBR1a different from GBR1b at that age.

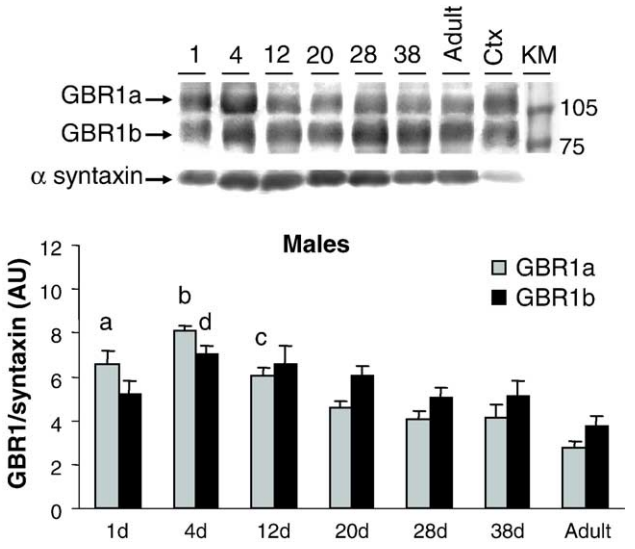

Fig. 2. GBR1 subunit expression in male hypothalami during development. Upper panel: representative Western blot of GBR1a/b isoforms: ages 1, 4, 12, 20, 28 and 38 days and adult rats. Lower panel: results in arbitrary units (AU) are the mean ± SE of 6–10 independent samples. Two-way ANOVA taking as factors GBR1 isoforms and age: interaction: $P < 0.02$, a: GBR1a different from adult, b: GBR1a different from 20 days onwards, c: GBR1a different from adult, d: GBR1b different from adult.

In males (Fig. 2), GBR1a and GBR1b expression was also higher in young animals and decreased gradually thereafter. Male rats showed adults levels for GBR1a at 20 days and for GBR1b at 12 days of age. No significant difference in the relative abundance of GBR1a with regard to GBR1b was observed in males during development.

We then compared levels of GBR1 variants in hypothalamic membranes between males and females. GBR1a expression was significantly higher in 1 day-old females while it was more abundant in males at 38 days (Fig. 3); no significant sex differences for GBR1b were observed at any age (Fig. 4).

GBR2 subunit was also detected in hypothalamic membranes of females and males at all ages studied (Fig. 5).

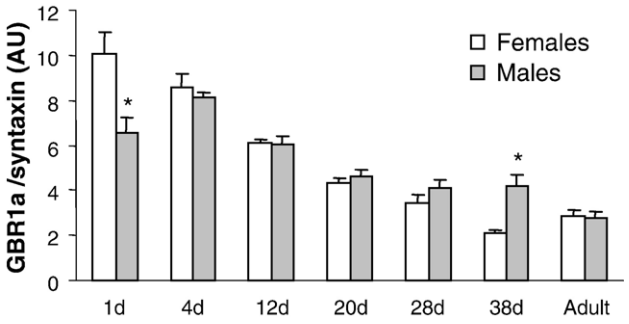

Fig. 3. Comparison of GBR1a isoform expression between male and female developing and adult rat hypothalamic membranes. Two-way ANOVA taking as factors sex and age: interaction: $P < 0.001$, *: significantly different from females at that age.

Maximum levels were observed at 12 postnatal days and adult levels were attained at 38 days; no sex differences were observed. Proestrous and lactating female hypothalamic levels of GBR2 were also similar [GBR2/α-syntaxin (AU): proestrous: 0.71 ± 0.06 (n = 6) vs. lactating: 0.67 ± 0.09 (n = 4), ns].

4. Discussion

Here, we describe for the first time the ontogenesis of $GABA_B$ receptor expression in hypothalami of female and male rats. It shows similarities and differences with the expression in other brain regions and with the anterior pituitary. In hypothalamus, a CNS region critically involved in pituitary control, the expression of both GBR1 and GBR2 was higher during the first postnatal days and decreased thereafter reaching adult levels between the third and fourth weeks of life; this pattern is similar to the one described in spinal cord [19,25]. In contrast, in cerebellum and cerebral cortex, GBR1, but not GBR2, decreased with age [19,25]. Our previous results in the adenohypophysis also show an ontogenic decrease of GBR1 expression in both sexes, though in that tissue there was very low GBR2 detectability [5].

It has been postulated that both GBR1 variants are differentially regulated in the brain [3], with GBR1a being more abundant in newborns and GBR1b more abundant in adulthood [13,14]; the same was described for cerebral cortex, but not cerebellum, spinal cord and midbrain [25]. In hypothalamus, GBR1a is higher in newborns while GBR1b is higher around puberty and thereafter; this difference is more noticeable in females, where it attains statistical significance. This expression pattern of both GBR1 variants in hypothalamus is similar to cortex and differs from the anterior pituitary, where GBR1a is always substantially more abundant than GBR1b. Interestingly, in the hypothalamus, as in the anterior hypophysis, a sexual difference in GBR1a expression at early stages of development was observed. This variant is significantly more abundant in 1-day-old females than in same age males. At 38 postnatal days, GBR1a was significantly more abundant in males mainly at the expense of a fall in the expression of this variant in females. This age coincides with puberty onset in our colony [6]. A decrease in hypothalamic GABA signaling has been shown to be necessary for the initiation of puberty to occur [35]. The sharp decrease in hypothalamic GBR1a subunit expression in females may be associated to this phenomenon. Similar GBR1a levels were observed in both sexes in adulthood. In contrast to GBR1a, no sex differences were observed in GBR1b expression throughout development. While the sex differences observed in the expression of GBR1a in whole hypothalami at early stages of development are relevant, a study of this expression in

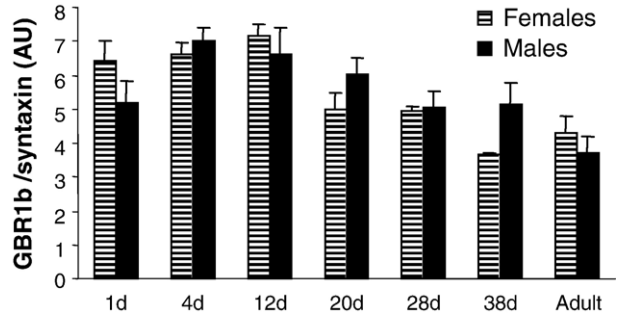

Fig. 4. Comparison of GBR1b isoform expression between male and female developing and adult rat hypothalamic membranes. Two-way ANOVA taking as factors sex and age: interaction: ns.

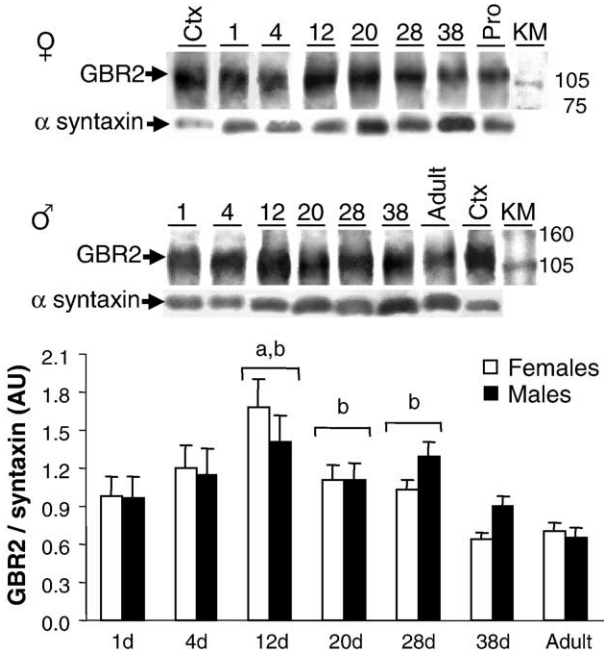

Fig. 5. GBR2 subunit expression in female and male hypothalami during development. Upper panel: representative Western blot of GBR2 subunit and the α-syntaxin control in female (♀) and male (♂) hypothalami: ages 1, 4, 12, 20, 28 and 38 days and adult rats. GBR2: bands immunostained with AbC22. Lower panel: Integration of GBR2 immunoblots relative to α-syntaxin controls. Two-way ANOVA taking as factors sex and age. Interaction: ns, main effect sex: ns, main effect age: $P < 0.01$, a: significantly different from 38 days; b: significantly different from adults.

discrete nuclei of the hypothalamus, especially in regions that subserve sex-specific functions, would clarify even further our understanding and will be addressed in future experiments.

GBR2 is clearly expressed in hypothalamus of developing rats, as has been described in adult animals [9]. In contrast to previous results in the anterior pituitary in which, although functional GBRs are present [23,24,33], we detected only GBR1 subunits [5], in the hypothalamus both GBR1 and GBR2 subunit are present consistent with the known expression of functional GBRs in this brain region [10,14,38].

Different to GBR1, GBR2 seems to attain maximal levels of expression at 12 days of age in both sexes. These results show that GBR1a, GBR1b and GBR2 have particular hypothalamic sex specific ontogenic expression patterns, suggesting that the expression of each isoform/subunit is under independent control during development, as was postulated during embryonic life in other brain areas [26]. The fact that the hypothalamic expression of GBRs is similar to some brain regions, as the spinal cord, but different from other CNS areas and from the pituitary, in both splice variant and subunit composition along development, suggests a specific role for each GBR composition in a particular location associated to a specific function.

It is tempting to speculate that if GABA is involved in the sexual differentiation of the brain [28] and $GABA_B$ receptors have been shown to play a role in the organization of the hypothalamic ventromedial nucleus [10], which is sexually dimorphic, i.e. larger in the adult male than female [27], and the fact that GBR1a subunits are significantly more abundant in hypothalami of females with regard to males at early stages of development, as determined in this work, then these GBR1a subunits may be involved in determining the sexually dimorphic development of this and/or other hypothalamic nuclei, in addition to other GABAergic mechanisms such as CREB phosphorylation through $GABA_A$ receptor activations [28]. This hypothesis will be the matter of future studies.

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