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REVIEW PAPER

$GABA_B$ Receptors in Neuroendocrine Regulation

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Abstract Gamma-amino butyric acid (GABA), in addition to being a metabolic intermediate and the main inhibitory neurotransmitter in the synaptic cleft, is postulated as a neurohormone, a paracrine signaling molecule, and a trophic factor. It acts through pre- and post-synaptic receptors, named $GABA_A$ and $GABA_C$ (ionotropic receptors) and $GABA_B$ (metabotropic receptor). Here we reviewed the participation of $GABA_B$ receptors in the regulation of the hypothalamic-pituitary-gonadal axis, using physiological, biochemical, and pharmacological approaches in rats, as well as in $GABA_{B1}$ knock-out mice, that lack functional $GABA_B$ receptors. Our general conclusion indicates that $GABA_B$ receptors participate in the regulation of pituitary hormone secretion acting both in the central nervous system and directly on the gland. PRL and gonadotropin axes are affected by $GABA_B$ receptor activation, as demonstrated in the rat and also in the $GABA_{B1}$ knock-out mouse. In addition, hypothalamic and pituitary $GABA_B$ receptor expression is modulated by steroid hormones. GABA participation in the brain control of pituitary secretion through $GABA_B$ receptors depends on physiological conditions, being age and sex critical factors.

These results indicate that patients receiving $GABA_B$ agonists/antagonists should be monitored for possible endocrine side effects.

Keywords $GABA_B$ receptors · Adenohypophysis · Hypothalamus · Pituitary hormones · $GABA_{B1}$ knock-out mice

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Introduction

The role of Gamma-amino butyric acid (GABA) as the predominant inhibitory neurotransmitter in the brain has been fully established. GABA is synthesized in 20–30% of all central nervous system neurons and participates in at least 40% of inhibitory synaptic processing. It is indispensable for the control of various functions such as locomotor activity, learning, and circadian rhythms (Bowery and Smart 2006; Krnjevic 2004).

Gamma-amino butyric acid synthesis is catalyzed by a 65-kDa and a 67-kDa form of glutamic acid decarboxylase (GAD) and catabolized by GABA transaminase. GABA and GAD are also found in ovaries, testis, fallopian tubes, liver, pituitary, kidney, adrenal glands, and insulin-producing β-cells of the pancreas (Fonnum 1987). The precise function of GABA in some of these tissues and cell types has yet

to be determined. In addition to acting as a neurotransmitter, GABA acts as a neurohormone, a paracrine signaling molecule, a metabolic intermediate, and a trophic factor (Beleboni et al. 2004; Bowery and Smart 2006; Lujan et al. 2005).

At the time the field of neuroendocrinology started to develop the importance of neurotransmitters in hormone release began to be established. The first reports, not always in agreement, indicated prolactin and LH were pituitary hormones under GABA regulation (Ondo 1974; Libertun and McCann 1976; Mioduszewsky et al. 1976). Later, different works postulated GABA as a neuroendocrine regulator, even before a clear picture of GABA receptors was elucidated (Ondo 1974; Libertun et al. 1979; Arakelian et al. 1984; Fiszer de Plazas et al. 1982, 1983).

GABA Receptors

The first of GABA receptors to be characterized was what we today know as the $GABA_A$ receptor (Bowery and Smart 2006). This is an ionotropic receptor, involving a chloride channel, associated with fast inhibitory conductance events. It presents a pentameric structure comprising a variety of possible combinations of protein subunits (Olsen and Tobin 1990). These receptors also have sites for positive allosteric modulation for different compounds such as benzodiazepines, anesthetics, neurosteroids (Haefely et al. 1975; Bowery and Smart 2006). Various agonists and antagonists for this receptor have been described. Similar to $GABA_A$ receptors, $GABA_C$ receptors represent a relatively simple form of transmitter-gated chloride channels made up of a single type of protein subunit, the Rho subunit (Chebib and Johnston 1999).

Regarding the effect of GABA on neuroendocrine regulation, a variety of effects on hormone secretion were described for its action on the $GABA_A$ receptor, acting both at the central nervous system and directly on the gland (Libertun and McCann 1976; Libertun et al. 1979; Fiszer de Plazas et al. 1983; Apud et al. 1989; Tuomisto and Mannisto 1985; McCann and Rettori 1986; Anderson and Mitchell 1986a, b; Roussel and Astier 1990; Lacau-Mengido et al. 1989; Virmani et al. 1990; Mannisto et al. 1992; Jarvinen et al. 1992; Seong et al. 1995).

In 1980 Bowery et al. first introduced the concept of a second, pharmacologically distinct GABA receptor named $GABA_B$ (Bowery et al. 1980). $GABA_B$ receptors are GABA and Baclofen (β(p-chlorophenyl GABA) sensitive; muscimol, bicuculine, and picrotoxin insensitive; located pre- and post-synaptically. They are metabotropic receptors, $G_{i/0}$-linked and therefore Pertussis Toxin (PTX) sensitive. $GABA_B$ receptor activation induce late, slow hyperpolarization, attenuation in membrane Ca^{2+} conductance (VSCC), increase in membrane K^+ conductance (Kir-3), inhibition of adenylyl cyclase (Bowery 1993). All these characteristics were discovered long before the molecular structure of the receptor was resolved. In addition, Baclofen was already in clinical use as a GABA-related compound for several years, for treatment of spasticity and other neurological disorders, without any knowledge of possible side effects on, for example, the endocrine system (Enna and Bowery 2004; Bowery 2006; Ulrich and Bettler 2007).

Soon after the first description of the existence of $GABA_B$ receptors we became interested in their possible participation in the neuroendocrine control of pituitary physiology.

$GABA_B$ Receptors in the Brain Control of Pituitary Secretion: Pharmacological Studies

Our first question was: Are $GABA_B$ receptors involved in the regulation of pituitary secretion? We tried to answer this hypothesis by performing a series of in vivo and in vitro experiments.

Effect of Baclofen In vivo on Basal and Stimulated Prolactin Release

First we investigated whether $GABA_B$ receptor stimulation modified PRL secretion. Baclofen did not alter basal PRL secretion but significantly decreased stress-stimulated PRL release by a variety of stressful stimuli such as ether, immobilization, swimming, or cold (D'Eramo et al. 1986), all described to induce the secretion of this hormone (Libertun et al. 1982). This effect did not depend on the endocrine status of the animal, as the response was the same in prepubertal or castrated adult males.

Similar results were also observed in prepubertal or castrated adult females. In addition, this inhibitory effect on stress-induced PRL release was dose-dependent (Fig. 1a).

Next, the effect of Baclofen was evaluated in another physiological model, i.e., when PRL release is stimulated by suckling (Arakelian and Libertun 1977; Leong et al. 1983). In this case, pre-injection with Baclofen completely abolished the expected PRL and TSH release when pups were restored to suckling dams (Lux et al. 1986). No effect was observed on LH secretion. No difference in milk content was observed, suggesting that Baclofen was not acting on oxytocin secretion (Fig. 1b). As both PRL and TSH are under TRH regulation during suckling, this neuropeptide was a possible site of action of the drug.

Interaction of Baclofen with Neurotransmitters and Neurohormones in Pituitary Secretion

Then we tried to determine which neural pathway/s were involved in $GABA_B$ receptor-mediated inhibition of PRL release. As dopamine is the main prolactin inhibiting hormone (Neill 1980, 1988; Libertun et al. 1982), we first evaluated if increased PRL due to dopamine receptor blockage with Haloperidol or inhibition of dopamine synthesis with α methyl-p-tyrosine were affected by Baclofen administration. The drug could not modify high PRL levels due to these drugs, possibly because the loss of dopaminergic input to the pituitary generates a very strong response in terms of PRL secretion.

Among the elusive PRL releasing factors, serotonin had been described to induce PRL release by

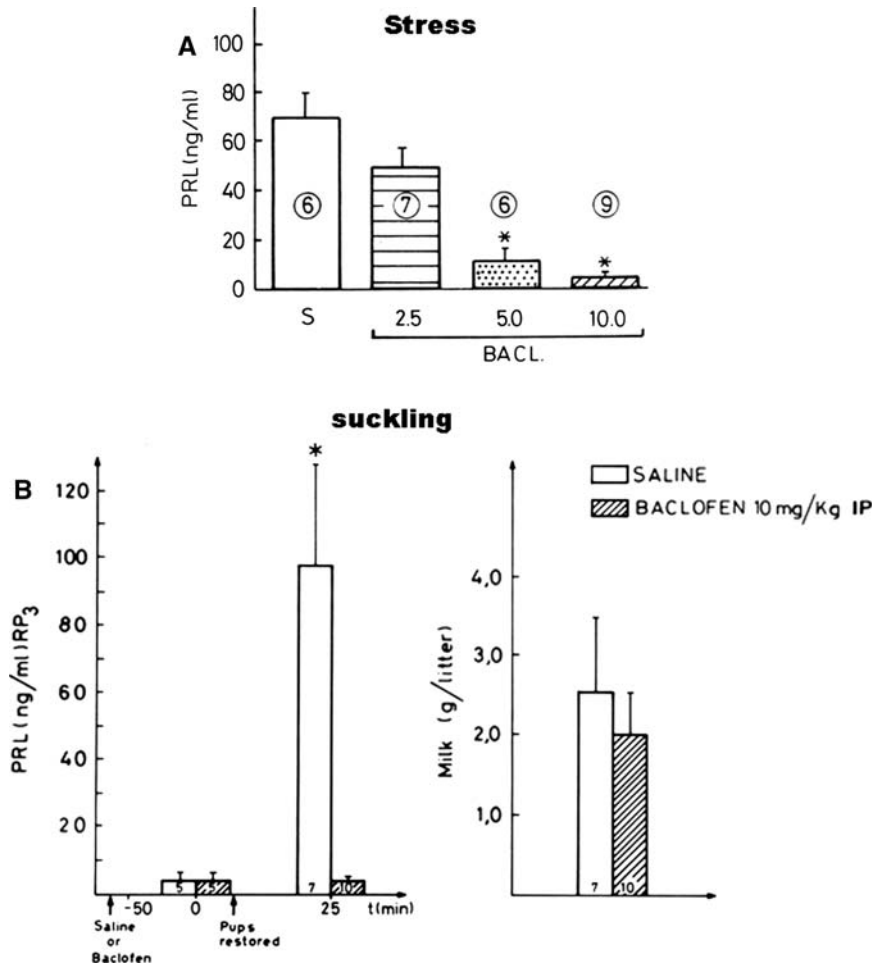

Fig. 1 Effect of Baclofen on stress and suckling-induced prolactin secretion. (**a**) Dose response effect of Baclofen (BACL) (2.5, 5, and 10 mg/kg) on prolactin secretion induced by immobilization stress in male rats. Numbers inside or above columns indicate number of rats per group. * $P < 0.05$. (Published in Proc Soc Exp Biol Med 197: 337–341:1991). (**b**) Effect of baclofen on PRL secretion and milk yied after 25 min suckling. Numbers inside columns indicate number of animals per group. * $P < 0.05$, compared to saline controls. Published in *Proc Soc Exp Biol Med* 183: 358–362:1986. Both figures are reproduced with the permission of the Society for Experimental Biology and Medicine

acting at the CNS (Becu-Villalobos et al. 1984; Pan 1991). Baclofen inhibited PRL release induced by this agent, suggesting that it could be interfering with serotonergic pathways. As TRH had also been involved in PRL secretion in situations of stress or suckling (Plotsky and Neill 1982; de Greef and Visser 1981; Johnston et al. 1984), we evaluated the effect of Baclofen under TRH administration. In this case, the drug inhibited PRL secretion but not TSH secretion. As TRH is acting directly on pituitary TRH receptors, one can assume that Baclofen does have an effect directly on the gland regulating PRL but not TSH secretion (Lux-Lantos et al. 1991).

Ontogenic Effect of Baclofen on PRL Secretion

The control of pituitary hormones secretion along ontogeny depends on the progressive development of the different neuroendocrine systems involved. In addition, $GABA_B$ receptors have been shown to be present in the brain already at early stages of development (Knott et al. 1993). We therefore decided to evaluate the effect of Baclofen on PRL release from birth to adulthood. Surprisingly, our results showed that Baclofen had a PRL-stimulating effect that increased from 4-day-old to 20-day-old animals (Rey-Roldán et al. 1997). At 28 days of life the effect of Baclofen on PRL secretion became transiently inhibitory and thereafter no action was observed on basal secretion, as demonstrated above. Another group described a similar response in 16- and 30-day-old female rats (Moguilevsky et al. 1992). This particular ontogenic pattern of Baclofen action on PRL release was sex independent, but hormone specific, as this $GABA_B$ agonist was devoid of action on TSH secretion at any stage of development and $GABA_B$ receptor activation was always inhibitory on LH secretion (Moguilevsky et al. 1991). $GABA_B$ receptors have been shown to modulate neurotransmitter systems which are involved both in inhibiting PRL secretion, such as dopamine (Santiago and Westerink 1992; Vacher et al. 2006), and in stimulating PRL secretion such as serotonin (Gray and Green 1999) (Racke et al. 1987). The balance of actions on these systems together with a direct action on the pituitary (see below) may account for this distinct pattern of PRL release. Interestingly, while the effect of Baclofen on PRL release was stimulatory, the co-administration of Baclofen with TRH induced an additive action, suggesting different mechanisms of action. When the stimulatory effect of Baclofen on PRL secretion was lost, Baclofen inhibited the TRH-induced PRL secretion.

Thus, conclusions from these in vivo experiments indicated that:

- $GABA_B$ receptors are involved in the regulation of pituitary hormones secretion.
- There is a characteristic ontogenic pattern of PRL response to $GABA_B$ receptor stimulation, being stimulatory until 20 days of age and inhibitory or devoid of effect on basal secretion thereafter.
- $GABA_B$ receptors participate in the regulation of PRL release induced by various stressful stimuli in adult rats. This response is not altered by the hormonal status of the animal.
- $GABA_B$ receptors also participate in the secretion of PRL and TSH induced by suckling, without altering the release of LH or oxytocin.
- Baclofen acts at the CNS inhibiting releasing factors involved in PRL and TSH secretion such as serotonin.
- Baclofen also blunts the TRH-induced PRL release, without affecting TSH secretion, probably through a direct action at the pituitary.

As these results suggested a possible site of action for Baclofen directly at the pituitary, we directed our investigations toward this aim.

Effect of Baclofen on In Vitro-Cultured Anterior Pituitary Cells

We performed anterior pituitary cell cultures from adult rats and studied the effect of Baclofen on hormone secretion. Baclofen significantly, and concentration dependently, inhibited basal PRL secretion (Lux-Lantos et al. 1992). This effect was reversed by specific antagonists (Fig. 2). In addition, Baclofen inhibited TRH-induced PRL secretion. The $GABA_B$ agonist also inhibited GnRH-induced LH secretion without modifying basal secretion. The same was observed for FSH. No effect was observed on TSH secretion, either basal or TRH-stimulated, in agreement with previous in vivo results with TRH administration (Rey-Roldán et al. 1997). Baclofen also had direct effects on hormone secretion from adenohypophyseal cells of immature rats, though

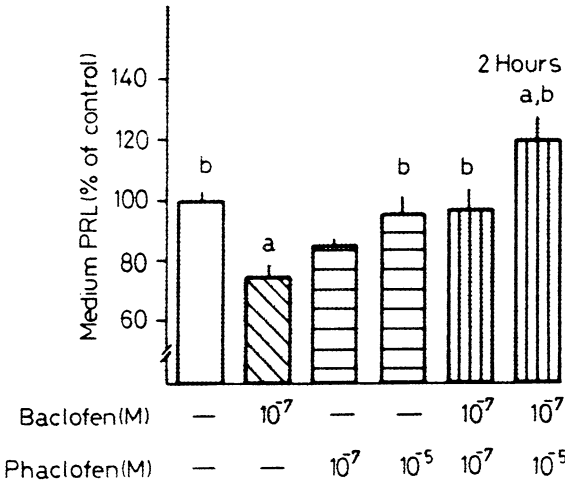

Fig. 2 Phaclofen antagonizes baclofen-induced PRL secretion inhibition in monolayer cultures of adenohypophyseal dispersed cells after 2-h incubation. Results are expressed as the percentage of control values, considering the control as 100%. a: significantly different from control values, b: significantly different from baclofen values. Published in *Neuroendocrinology* 56:687–693:1992. Reproduced with the permission of S. Karger AG, Basel

somewhat different from those observed in adults (Rey-Roldán et al. 1996). These differences probably depended on the stage of development of the neuroendocrine controls of each axis. In 12-day-old female rats, when the pituitary is highly sensitive to stimulatory inputs (Becu-Villalobos et al. 1990, 1997), Baclofen significantly inhibited GnRH-induced LH and FSH release, without affecting either basal or TRH-stimulated PRL or TSH secretion.

So we demonstrated for the first time a direct action of Baclofen on pituitary secretion, suggesting the presence of $GABA_B$ receptors at least in some pituitary cell types. Whether the effects observed where of an autocrine or paracrine nature remained to be established.

Our next aim was the study of the mechanism of action of Baclofen at the pituitary.

$GABA_B$ Receptor Activation in Anterior Pituitary Cells. Participation of Gi/o Proteins and Intracellular Signaling

First we showed that PTX completely inhibited the effect of Baclofen on hormone secretion in pituitary cell cultures, suggesting the participation of Gi/0 proteins (Lux-Lantos et al. 2001). As central $GABA_B$ receptors had been demonstrated to be negatively coupled to adenylyl cyclase, we then studied the effect of Baclofen on cAMP production in pituitary cells. Forskolin, a stimulator of adenylyl cyclase, induced an increase in cAMP levels. This increase was significantly inhibited by Baclofen and the action of Baclofen was reversed either by pre-incubation with PTX or by the addition of the $GABA_B$ receptor antagonist 2-hydroxysaclofen (Fig. 3). Therefore we demonstrated that $GABA_B$ receptors are also negatively coupled to adenylyl cyclase in pituitary cells, involving a Gi/0 protein.

As $GABA_B$ receptors had also been postulated to inhibit calcium channels through the $\beta\gamma$ subunit of the G protein, we evaluated the effect of Baclofen on intracellular calcium titers. The drug decreased basal calcium levels without affecting TRH-induced calcium increase. This effect was much weaker than under dopamine stimulation. We next tried to determine which pathways were involved in the calcium lowering effect when $GABA_B$ receptors were activated. On the one hand, this effect was completely abolished by pre-incubation of VGCC blockers such as Nifedipine or Verapamil. On the other hand, pre-incubation with K^+ channel blockers such as 3-ethylamonium or Barium Chloride did not impair the effect of Baclofen on intracellular calcium titers. In addition membrane depolarization with high K^+ again did not compromise Baclofen action. These results suggested an action

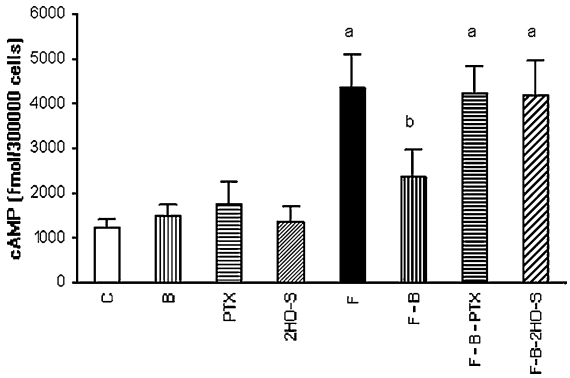

Fig. 3 Effect of baclofen on forskolin-induced cAMP production in anterior pituitary cells from proestrous rats. C: control, B: baclofen 1.10^{-5} M, PTX: Pertussis Toxin (150 ng/ml, 20 h), 2HO-S: 2 hydroxysaclophen 1.10^{-4} M, F: forskolin 0.5 μM. a: significantly different from control values, b: significantly different from F, F-B-PTX, and F-B-2HO-S. n: four experiments. Published in *Neuroendocrinology* 73: 334–343:2001. Reproduced with the permission of S. Karger AG, Basel

though calcium channels, as had been suggested for $GABA_B$ receptor activation at pre-synaptic sites, inhibiting neurotransmitter release (Lux-Lantos et al. 2001). Similar results were obtained by another laboratory working on $GABA_B$ receptors in neurointermediate lobe melanotropes, supporting our observations (Shibuya et al. 1999; Shibuya and Douglas 1993).

Interestingly, in preliminary experiments we determined that the effect of Baclofen on intracellular calcium in pituitary cells was lost if female rats had previously been injected with estradiol; in later experiments this observation would be investigated in depth (see below).

So the conclusions from evaluating functionality and mechanism of action of $GABA_B$ receptors in anterior pituitary cells were:

- Indirect demonstration of the presence of $GABA_B$ receptors in anterior pituitary cells.
- Inhibition of basal and TRH-stimulated PRL secretion by $GABA_B$ agonists in pituitary cells from adult animals. Specificity of effects, as they were reversed by specific antagonists.
- Inhibition of GnRH-stimulated LH and FSH secretion in pituitary cells from adult and infantile animals.
- No effect was observed on TSH secretion either basally or under TRH stimulation.
- Baclofen effects were abolished by pre-incubation with PTX, suggesting the participation of Gi/0 proteins in the signaling pathway.
- $GABA_B$ receptor activation inhibits Forskolin-induced cAMP production, indicating negative coupling to adenylyl cyclase.
- $GABA_B$ receptor activation inhibits intracellular Ca^{2+} levels. This involves closure of VGCC.
- The mechanism of action of $GABA_B$ receptor stimulation at the pituitary is similar to the one described at the CNS in pre-synaptic sites.

$GABA_B$ Receptor Structure: Ontogenic Expression and Regulation of Expression of $GABA_B$ Receptors in the Hypothalamic-Pituitary Unit

After remaining elusive for some time, finally the molecular structure of $GABA_B$ receptors was discovered. First in 1997, the group of Dr. B. Bettler cloned a $GABA_B$ receptor which turned out to belong to the 7-transmembrane domain (7TM) receptors coupled to G proteins (Kaupmann et al. 1997). However, when this receptor was expressed in cells it did not function as predicted. One year later the same group, as well as others, demonstrated that the receptor was a heterodimer formed by two dissimilar 7TM subunits (Kaupmann et al. 1998; White et al. 1998; Kuner et al. 1999). $GABA_{B1}$ provides the GABA-binding domain while $GABA_{B2}$ provides G-protein coupling (Couve et al. 2004; Bettler and Tiao 2006). Soon thereafter Bettler et al. developed antibodies against each of the subunits of the $GABA_B$ receptor and demonstrated that the $GABA_{B1}$ subunit exists in two main functional isoforms, $GABA_{B1a}$ of 120 KDa and $GABA_{B1b}$ of 100 KDa, dimerizing with the $GABA_{B2}$ subunit of 110 KDa (Kaupmann et al. 1998). Additional splice variants of $GABA_{B1}$ have since been identified (Isomoto et al. 1998; Pfaff et al. 1999; Schwarz et al. 2000), though their physiological importance has not been elucidated.

In view of the new knowledge on the receptor structure, we decided to study the expression of these subunits in the hypothalamic-pituitary unit, in collaboration with Dr. Bettler.

GABA and its receptors have particular ontogenic distributions in different rat brain areas. 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 (Turgeon and Albin 1994; Bowery 1996; Bowery et al. 2002); others have described that they either decrease or do not change along ontogeny, depending on the region of the nervous system analyzed (Malitschek et al. 1998). In some cases, changes in the relative expression of receptor splice variants have also been reported (Fritschy et al. 1994, 1999, 2004; Malitschek et al. 1998).

Ontogenic Expression of $GABA_B$ Receptors in the Hypothalamic-Pituitary Unit

We determined the ontogenic expression of the $GABA_{B1}$ and $GABA_{B2}$ subunits of the $GABA_BR$ in the hypothalamus. Western blots analysis showed that both, $GABA_{B1}$ and $GABA_{B2}$, were expressed in male and female hypothalamic membranes from day

1 to adulthood (Bianchi et al. 2005). In females, both $GABA_{B1a}$ and $GABA_{B1b}$ were maximally expressed in newborns and decreased toward adulthood. At birth, expression of $GABA_{B1a}$ was significantly higher than $GABA_{B1b}$, while at 38 days $GABA_{B1b}$ was more abundant. In males, $GABA_{B1a}$ and $GABA_{B1b}$ 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 $GABA_{B1}$ variants levels in hypothalamus between sexes, $GABA_{B1a}$ was transiently and moderately more abundant in females at birth while at 38d its expression was higher in males; $GABA_{B1b}$ showed no sex differences along development. $GABA_{B2}$ 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.

The hypothalamic ontogenic expression of the $GABA_BR$ shows similarities and differences with other brain regions; this pattern was similar to the one described in spinal cord (Malitschek et al. 1998; Kaupmann et al. 1998). In contrast, in cerebellum and cerebral cortex $GABA_{B1}$, but not $GABA_{B2}$, decreased with age (Malitschek et al. 1998; Kaupmann et al. 1998). In addition, it has been postulated that both $GABA_{B1}$variants are differentially regulated in the brain (Benke et al. 1999). In hypothalamus $GABA_{B1a}$ was more abundant in newborns while $GABA_{B1b}$ was higher around puberty and thereafter, as was described for cerebral cortex, but not cerebellum, spinal cord, and midbrain (Malitschek et al. 1998); this difference was more noticeable in females. These results show that $GABA_{B1a}$, $GABA_{B1b}$, and $GABA_{B2}$ 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 (Martin et al. 2004). In addition, it suggests a specific role for each $GABA_BR$ composition in a particular location associated to a specific function.

Our novel results demonstrated the ontogenic expression of the $GABA_B$ receptor subunits in anterior pituitaries of male and female rats. Both isoforms of the $GABA_{B1}$ subunit decreased with age in female pituitaries. $GABA_{B1a}$ was significantly more expressed than $GABA_{B1b}$ in the neonatal and infantile periods, while at adulthood this difference did not quite attain statistical significance. The $GABA_{B2}$ subunit was hardly detectable in anterior pituitary membranes of females at all ages (Bianchi et al. 2001). In males, $GABA_{B1a}$ also decreased along development; $GABA_{B1b}$ and $GABA_{B2}$ were barely detectable. When comparing expression levels of $GABA_{B1a}$ between sexes, we observed a very significant difference at early stages of development, being this expression increased in females (Fig. 4). Binding studies with ^{3}H-Baclofen on pituitary membranes from males and females largely confirmed the above findings.

The decrease of $GABA_{B1}$ expression along development was similar to our observations in hypothalamus; on the other hand, in contrast to hypothalamus and many other brain regions, in the pituitary $GABA_{B1a}$ was the most abundant isoform at all the ages studied.

The significant difference in intensity of $GABA_{B1a}$ expression between females and males and the lack of $GABA_{B1b}$ expression in males suggests a sexually dimorphic expression of $GABA_B$ subunits during ontogeny in the rat pituitary. Sexual differences in pituitary function and in central nervous system structures involved in the control of the adenohypophyseal secretion have been extensively described (Becu-Villalobos and Libertun 1995; Becu-Villalobos et al. 1997) and point to the critical role of neonatal sexual steroids in these events.

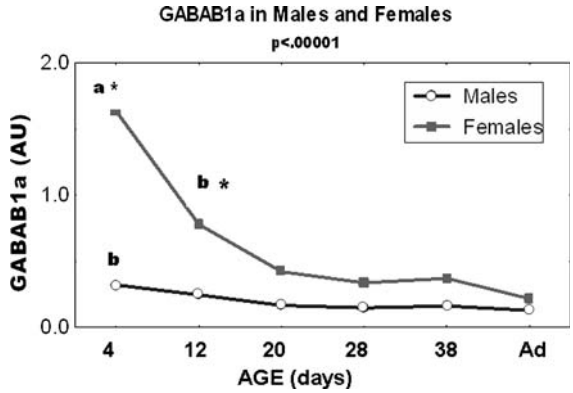

Fig. 4 Integration of $GABA_{B1a}$ receptor subunit inmunoblots of female and male developing and adult rat anterior pituitary membranes. Results in arbitrary units (AU) are the mean ± SE of 4–5 independent samples and are expressed as the relation of each subunit with regard to the syntaxin control. a: significantly different from 12 days onwards; b: significantly different from 20 days onwards. * significantly different from males at a certain age. For all cases: $P < 0.05$ or less

Influence of Perinatal Testosterone on Pituitary $GABA_{B1}$ Subunit Expression

In rats there is a sensitive developmental period during which sexual differentiation of neural substrates proceeds irreversibly under the influence of gonadal hormones. This period starts a few days before birth and ends approximately 10 days thereafter (Gorski 2002).

As males present two testosterone peaks, one on embryonic day 18 and one on the first day of life, we hypothesized that these could be involved in the sexually dimorphic expression of $GABA_{B1}$ subunit of the $GABA_BR$ observed in the pituitary.

To this aim, different in vivo treatments were undertaken. In Fig. 5 we show the expression of both isoforms of the $GABA_{B1}$ subunit in 8-day-old females, 8-day-old females treated neonatally with two different doses of testosterone propionate, which have been shown to effectively androgenize females, 8-day-old males, 8-day-old males castrated neonatally, in which the second testosterone androgenizing peak was abolished, 8-day-old males treated *in utero* with flutamide, an antiandrogen that abolishes the first androgenizing peak, and 8-day-old males treated with flutamide and castrated on the day of birth (elimination of both testosterone peaks). Western blots showed that females have higher levels of expression of both isoforms of the $GABA_{B1}$ subunit than males of the same age, as demonstrated before. High-dose testosterone treatment decreased levels of expression in females to male levels, while flutamide or flutamide plus castration increased levels of expression in males to female levels. These results were confirmed by RT-PCR studies. Therefore androgens acting pre- and post-natally decrease the expression of the pituitary $GABA_{B1}$ subunit of the $GABA_B$ receptor (Bianchi et al. 2004) and seem to originate the sexually dimorphic expression observed. The mechanism by which this effect is produced is still under investigation.

The evidence of steroid-dependent sexual differences in $GABA_BR$ expression is not an isolated observation, since the participation of GABA and its receptors has been proposed to play key roles in sexual differentiation of the brain and in sexually dimorphic hormone secretion patterns (Davis et al. 1996; Murray et al. 1999; Davis et al. 2000). Continuing with the hypothesis that gonadal steroids influenced $GABA_BR$ expression, we evaluated the effect of estradiol on this parameter.

Effect of Estradiol on $GABA_B$ Receptor Expression in the Hypothalamus and Pituitary of Adult Female Rats

In view of the above-mentioned results, pituitary $GABA_B$ receptor expression seemed to be under androgen regulation. Interestingly, preliminary experiments had suggested that estrogens could also participate in the regulation of $GABA_B$ receptors expression and/or function. We had observed that the effect of Baclofen on intracellular calcium was lost in pituitary cells when females had been injected with estrogens. To confirm this hypothesis, the expression of $GABA_B$ receptors in the hypothalamic-pituitary unit was determined in estrogenized female rats in comparison to proestrous rats. At the pituitary, the long-term (5 weeks), but not the short-term (1 week), treatment with estradiol significantly decreased the expression of both subunits of the $GABA_B$ receptor determined by RT-PCR. At the hypothalamus the long-term treatment decreased both subunits while the short-term treatment only reduced the $GABA_{B2}$ subunit mRNA expression. This was also confirmed at the protein levels by Western blots (Rey-Roldan et al. 2006). This decrease in receptor expression was accompanied by a decrease in receptor function, as Baclofen was unable to modify calcium levels in long-term estrogenized female pituitary cells. This lack of response is probably due to estradiol-induced downregulation of $GABA_BRs$, 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 (Livingstone et al. 1998; Kelly and Wagner 1999), all factors involved in the $GABA_BR$ signaling pathway.

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.

Conclusions from $GABA_B$ receptor subunit expression are:

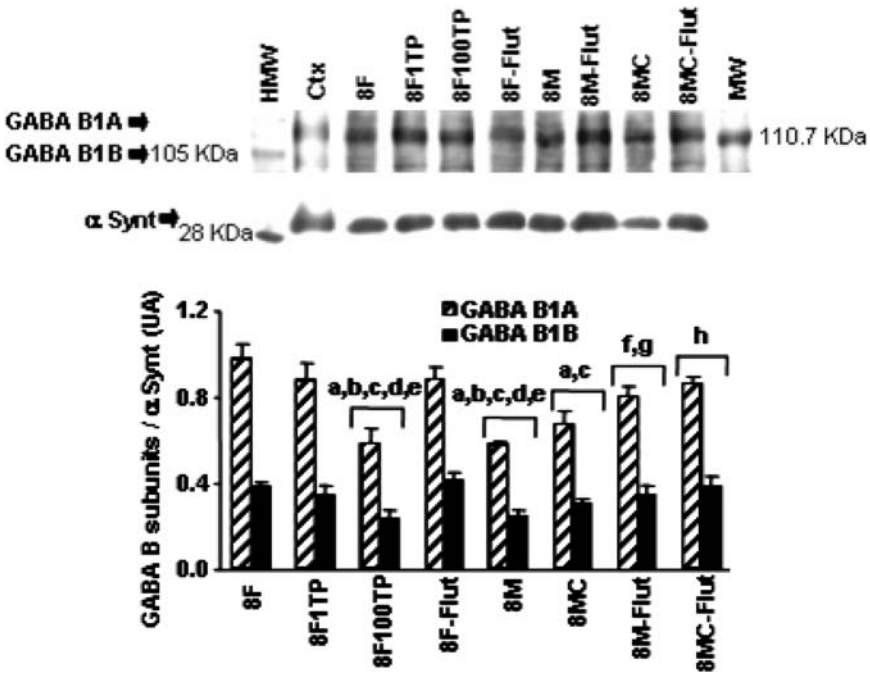

Fig. 5 $GABA_{B1}$ receptor subunit expression in anterior hypophysis from 8-day-old animals under different treatments determined by Western Blot with specific antisera. 8F: 8-day-old control females, 8F1TP: 8-day-old females injected with 1 μg TP on days 1–4 of life, 8F100TP: 8-day-old females treated with 100 μg of TP on the day of birth, 8F-Flut: 8-day-old female pups from Flutamide injected mothers, 8M: 8-day-old control males, 8MC: 8-day-old males castrated on the day of birth, 8M-Flut: 8-day-old males from Flutamide injected mothers, 8MC-Flut: 8-day-old males from Flutamide injected mothers and castrated on the day of birth. Upper panel: Representative Western Blot for $GABA_{B1a}$ and $GABA_{B1b}$ subunit and α syntaxin expression. Ctx: cortex membranes used as positive controls. MW: molecular weight markers. Lower panel: Data are expressed as the ratio of $GABA_{B1}$ subunit expression to α syntaxin expression. Number of samples per group: 8F = 12, 8F1TP = 6: 8F100TP = 12, 8F-Flut = 6, 8M = 12, 8MC = 12, 8M-Flut = 6 and 8MC-Flut = 10. Results were analyzed by two-way ANOVA: interaction: ns, indicating that both splice variants follow the same pattern of expression, factor subunit: $P < 0.001$, indicating that $GABA_{B1a}$ is always more abundant than $GABA_{B1b}$, factor treatment: $P < 0.001$ or less; a: different from 8F, b: different from 8F1TP, c: different from 8F.Flut, d: different from 8M-Flut, e: different from 8MC-Flut, f: different from 8F100TP, g: different from 8M, h: different from 8MC. Published in *Neuroendocrinology* 80:129–142:2004. Reproduced with the permission of S. Karger AG, Basel

- The expression of hypothalamic and anterior pituitary $GABA_B$ receptor subunits displays a particular ontogenic pattern being, in general, levels high at birth and decreasing toward adulthood in both sexes.
- Hypothalamic and pituitary $GABA_{B1}$ subunits present a sexually dimorphic expression, being neonatally significantly more expressed in females than in males. This difference is more marked in the pituitary.
- Perinatal testosterone seems responsible for the lower $GABA_{B1}$ expression levels in the adenohypophysis of neonatal males.
- In the adult female long-term estradiol treatment downregulates $GABA_B$ receptor expression in the hypothalamus and the pituitary.
- Thus, $GABA_B$ receptor expression is modulated by gonadal steroid hormones.

Neuroendocrine Studies in $GABA_{B1}$ Knock-Out Mice

In the last few years Dr. Bettler's group developed two strains of mice deficient in either the $GABA_{B1}$ subunit (Schuler et al. 2001) or the $GABA_{B2}$ subunit (Gassmann et al. 2004), both of which suffer from spontaneous seizures, hyperalgesia, hyperlocomotor activity and severe memory impairment, and, upon administration of a $GABA_B$ agonist, absence of typical $GABA_B$ responses such as muscle relaxation, hypothermia, and delta EEG waves. This demonstrates that

most $GABA_B$ functions depend on heterodimerization between the $GABA_{B1}$ and $GABA_{B2}$ subunits. However, $GABA_{B2}$KO, but not $GABA_{B1}$KO mice, still exhibit atypical electrophysiological $GABA_B$ responses, indicating that, in vivo, $GABA_{B1}$, but not $GABA_{B2}$, can be functional in the absence of the partner subunit (Gassmann et al. 2004). More recently still, knock-out mice of each of the $GABA_{B1}$ isoforms, $GABA_{B1a}$ and $GABA_{B1b}$, have been developed by the same group, pointing to specific roles for receptor subtypes (Ulrich and Bettler 2007).

In this way a new tool came into our hands to continue our studies on the participation of $GABA_B$ receptors in endocrine function, the $GABA_{B1}$KO mice, lacking the expression of both isoforms of the $GABA_{B1}$ subunit.

Our First Question Was: Do $GABA_{B1}$ Knock-Out Mice Show Endocrine Alterations?

We first evaluated pituitary weight and pituitary hormones serum titers in both sexes and both genotypes and found no significant differences between genotypes in most hormones, such as LH, FSH, TSH, and GH, in basal conditions. The only difference observed in these hormones were sex-related, with female mice showing larger pituitaries and male mice showing higher FSH, LH, and TSH serum levels (Catalano et al. 2005).

Prolactin

PRL was the only pituitary hormone assayed which was basally altered in $GABA_{B1}$KO mice, exclusively in males. Females of both genotypes showed similar PRL titers, significantly higher than wild-type (WT) males. In contrast, $GABA_{B1}$KO males showed significantly higher PRL than WT controls, similar to female titers. As PRL was altered in $GABA_{B1}$KO males we investigated whether this alteration was also evidenced when PRL was stimulated by immobilization stress. This was not the case; the increase in PRL release induced by stress was similar between genotypes. In addition, immobilization stress was proposed to reduce TSH levels in rodents (Ruisseau et al. 1978; Marti et al. 1996); this effect was observed but, again, without differences between genotypes (Catalano et al. 2005).

Estrous cyclicity and reproductive function in $GABA_{B1}$KO mice

Although we did not find differences in basal gonadotropin levels or in puberty parameters between genotypes, GABA through both its receptors has been related to gonadotropin regulation. We therefore evaluated cyclicity in females from both genotypes. Estrous cycles were significantly disrupted in $GABA_{B1}$KO females. These animals showed longer periods in estrus, with shorter periods in proestrus (Catalano et al. 2005) (Fig. 6).

The results on estrous cycles, together with the observation in our colony that $GABA_{B1}$KO females did not get easily pregnant prompted us study this in more detail. To this aim we put one WT and one $GABA_{B1}$KO female in a cage with a male of known fertility and determined the number of pregnant females of each genotype in the first 30 days of male exposure. We also evaluated number of days to first delivery and litter size. Significantly fewer $GABA_{B1}$KO females got pregnant in the first 30 days of male exposure and there was also an increase in the days until delivery, though not attaining statistical significance. No difference in the number of pups per litter was detected between genotypes.

However, in the above set of experiments the difference in the pregnancy index could not discriminate between a possible lack of mating behavior, length of time in getting into proestrous or pregnancy interruption. Therefore, a new group of females from both genotypes was cycled and, on the day of proestrus, they were mated with a male of known fertility; the next day, the presence of the vaginal plug, indicative of positive mating behavior, was recorded. All WT and $GABA_{B1}$KO females mated on proestrous. Nevertheless, a significant difference in successful pregnancies was observed between genotypes (Catalano et al. 2005). This experiment demonstrated that even though $GABA_{B1}$KO females display alterations in estrous cycles, mating behavior is normal once they come into proestrus, yet at some later point fertility problems arise, which remain to be determined.

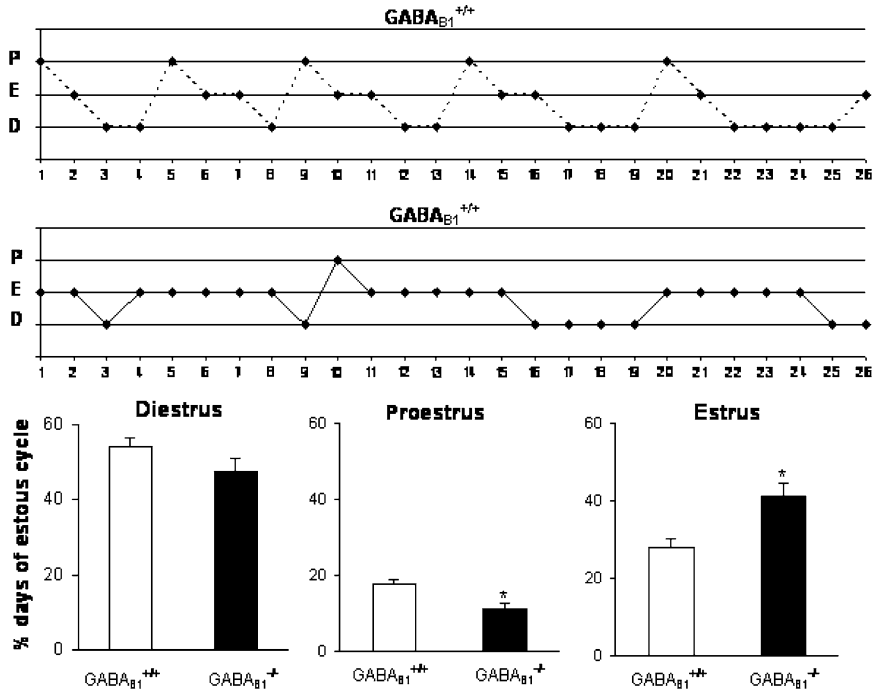

Fig. 6 Representative profiles illustrating the disruption of estrous cyclicity in $GABA_{B1}^{-/-}$ mice compared to wild-type (WT) controls, $GABA_{B1}^{+/+}$ (upper panel). Days in each phase of the estrous cycle (%) in $GABA_{B1}^{+/+}$ and $GABA_{B1}^{-/-}$ mice (lower panel). % days of estrous cycle: Student's t test. * $P < 0.001$, significantly different from WT mice. Published in *Neuroendocrinology* 82:294–305:2005. Reproduced with the permission of S. Karger AG, Basel

Post-Castration Gonadotropin Increase in Male and Female $GABA_{B1}$KO and Wild Mice

As cyclicity and reproductive functions were altered in female $GABA_{B1}$KO mice, we next evaluated the functionality of the gonadotropin axis in females. We determined LH and FSH in female mice in basal conditions, 7 days after ovariectomy and with the reinstatement of the estradiol negative feedback action by estradiol administration. We observed no differences between genotypes in either LH or FSH titers (Catalano et al. 2005). But as $GABA_A$ and $GABA_B$ receptors were suggested to be involved in the late increase in LH in female rodents after castration as compared to males (Gay and Midgley 1969; Luderer and Schwartz 1994; Yoo et al. 2000; Hood and Schwartz 2000), we evaluated this response in $GABA_{B1}$KO animals. LH rose significantly already on day 1 post-castration in males and remained elevated thereafter, without differences between genotypes. The LH rise was significantly delayed in females with regards to males. In WT females LH started to increase on day 7 post-castration. In KO females a significant advance in the LH rise was observed. On day 5, when in WT serum LH was still unchanged, in $GABA_{B1}$KO females this hormone reached 8-fold titers, suggesting that the absence of $GABA_{B1}$ receptors was involved in this advance in LH release. This effect was specific for LH as no differences in the post-castration FSH rise were observed between genotypes in either sex (Catalano et al. 2005).

Conclusions from the Evaluation of the Hypothalamic-Pituitary-Gonadal Axis in Male and Female $GABA_{B1}$KO and WT Mice

Our main conclusions from the evaluation of the hypothalamic-pituitary-gonadal axis in male and female $GABA_{B1}$KO mice are:

- $GABA_{B1}$KO males are hyperprolactinemic.
- $GABA_B$ receptors are not involved in stress-induced PRL release in mice.
- $GABA_{B1}$KO females show alterations in the gonadotropic axis including disruption of cyclicity, impaired fertility and advanced post-castration LH increase.

These results warrant further studies to determine the neuroendocrine pathways that are disturbed by the absence of $GABA_B$ receptors or if these effects are

the consequence of absence of these receptors in target organs such as the pituitary or the gonads.

General Conclusions.

- $GABA_B$ receptors participate in GABA regulation of pituitary function both in the central nervous system and directly at the gland.
- The PRL and gonadotropin axes are affected by $GABA_B$ receptor activation, as demonstrated by pharmacological and biochemical approaches in the rat, as well as in the $GABA_{B1}$KO mouse.
- Hypothalamic and pituitary $GABA_B$ receptor expression show specific ontogenic patterns and are modulated by steroid hormones.
- GABA participation in brain control of pituitary secretion through $GABA_B$ receptors is age and sex dependent and varies according to the physiological conditions.

These results indicate that patients receiving $GABA_B$ agonists/antagonists should be monitored for eventual endocrine side effects.

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