Reproductive Biology and Endocrinology

Research

Open Access

Stimulation of TM3 Leydig cell proliferation via $GABA_A$ receptors: A new role for testicular GABA

Christof Geigerseder, Richard FG Doepner, Andrea Thalhammer,
Annette Krieger and Artur Mayerhofer*

Address: Anatomisches Institut der Ludwig-Maximilians-Universität München, Biedersteinerstr.29, D-80802 München, Germany

Email: Christof Geigerseder - geigerseder@web.de; Richard FG Doepner - richard.doepner@lrz.uni-muenchen.de;
Andrea Thalhammer - andreathalhammer@t-online.de; Annette Krieger - annette.krieger@lrz.uni-muenchen.de;
Artur Mayerhofer* - Mayerhofer@lrz.uni-muenchen.de

* Corresponding author

Published: 24 March 2004

Reproductive Biology and Endocrinology 2004, **2**:13

This article is available from: http://www.rbej.com/content/2/1/13

Received: 11 December 2003
Accepted: 24 March 2004

Abstract

The neurotransmitter gamma-aminobutyric acid (GABA) and subtypes of GABA receptors were recently identified in adult testes. Since adult Leydig cells possess both the GABA biosynthetic enzyme glutamate decarboxylase (GAD), as well as $GABA_A$ and $GABA_B$ receptors, it is possible that GABA may act as auto-/paracrine molecule to regulate Leydig cell function. The present study was aimed to examine effects of GABA, which may include trophic action. This assumption is based on reports pinpointing GABA as regulator of proliferation and differentiation of developing neurons via $GABA_A$ receptors. Assuming such a role for the developing testis, we studied whether GABA synthesis and GABA receptors are already present in the postnatal testis, where fetal Leydig cells and, to a much greater extend, cells of the adult Leydig cell lineage proliferate. Immunohistochemistry, RT-PCR, Western blotting and a radioactive enzymatic GAD assay evidenced that fetal Leydig cells of five-six days old rats possess active GAD protein, and that both fetal Leydig cells and cells of the adult Leydig cell lineage possess $GABA_A$ receptor subunits. TM3 cells, a proliferating mouse Leydig cell line, which we showed to possess $GABA_A$ receptor subunits by RT-PCR, served to study effects of GABA on proliferation. Using a colorimetric proliferation assay and Western Blotting for proliferating cell nuclear antigen (PCNA) we demonstrated that GABA or the $GABA_A$ agonist isoguvacine significantly increased TM3 cell number and PCNA content in TM3 cells. These effects were blocked by the $GABA_A$ antagonist bicuculline, implying a role for $GABA_A$ receptors. In conclusion, GABA increases proliferation of TM3 Leydig cells via $GABA_A$ receptor activation and proliferating Leydig cells in the postnatal rodent testis bear a GABAergic system. Thus testicular GABA may play an as yet unrecognized role in the development of Leydig cells during the differentiation of the testicular interstitial compartment.

Background

Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the vertebrate central nervous system. In addition to its well established function as neurotransmitter, locally synthesized GABA and GABA receptors are also present in endocrine organs, for example, in somatotrophs (GH-producing cells) of the anterior pituitary lobe [1-3] and in pancreatic islet cells [4-6]. In both endocrine tissues GABA is regulating the synthesis and the release of hormones. The release of glucagon and growth hormone was shown to be controlled by GABA in an auto-/paracrine manner.

In our previous work we recently identified another GABAergic system located in adult Leydig cells in rodent and human testis [7]. Since Leydig cells possess both isoforms of the GABA synthesizing enzyme glutamate decarboxylase, GAD65 and GAD67, vesicular GABA transporter (VGAT), as well as several $GABA_A$ and $GABA_B$ receptor subunits, GABA may act as an auto-/paracrine molecule regulating Leydig cell function. Some evidence for a role in release of testosterone came from pharmacological studies in rat Leydig cells, which respond to GABAergic stimulation with increased testosterone production [8,9]. What other roles GABA may have in endocrine Leydig cells and which GABA receptors are mediating these actions are not known.

In the central nervous system evidence for a non-synaptic, trophic role of GABA in neurogenesis during embryonic development is mounting [10-15]. Thus GABA stimulates progenitor cells to proliferate in different regions of the developing brain [16-20]. Since these neuronal progenitor cells are also capable of synthesizing GABA and possess GABA receptors, GABA executes this trophic function in an auto-/paracrine fashion [21]. Further non-synaptic actions of GABA in the developing brain that are evolving include regulation of migration and motility of embryonic neurons [22-24]. While in general, cellular responses to GABA are mediated through $GABA_A$, $GABA_B$ and $GABA_C$ receptors and the intracellular signaling pathways associated with them [25], in respect to both cell proliferation and migration in the developing brain, contribution of $GABA_A$ receptors was reported [18,19,21,26,27]. Thus, although its precise regulation may depend on the region and cell type affected, GABA emerges as an important signal for cell proliferation and migration.

In view of this role of GABA in the brain, the question arises, whether GABA may influence cell proliferation processes in the testis, for example in Leydig cells, which bear GABA receptors [7]. In the testis of adult mammals, however, Leydig cells have only a marginal turnover rate and show low mitotic activity [28-30]. Due to the fact that Leydig cells in postnatal testis proliferate to a much greater extend than in adult testis [31-35], we sought to study postnatal testes of mice and rats at age of five-six days after birth. At this point of development two distinct types of Leydig cells are found, namely steroidogenic fetal Leydig cells with a typical rounded morphology clustered together in groups and spindle-shaped mesenchymal precursor cells of adult Leydig cells, which are located primarily in peritubular regions. The latter are not able to synthesize steroids, but are strongly proliferating and differentiate to Leydig progenitor cells during the second postnatal week in rodents [36-38]. Fetal Leydig cells may also increase in number during postnatal development, albeit to a smaller degree [31,39]. Thus, the endocrine compartment of postnatal testis bears developing and highly proliferating cells of the adult Leydig cell lineage. Therefore, in this study we addressed the questions whether a local GABAergic system is present in postnatal testis and may be involved in proliferation of Leydig cells.

Methods

Animals

Testes and other tissues were obtained from adult, 3–6 months old (n = 12; Sprague-Dawley, Wistar) and five-six days old male rats (n = 14; Sprague-Dawley), as well as from adult (n = 4; BALB/c) male mice, which were bred at the Technische Universität München, Germany. Testes were also obtained from five-six days old male mice (n = 9; BALB/c), which were bred at the Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina. According to the National Institute of Health Guide for the Care and Use of Laboratory Animals, they were painlessly killed under ether anesthesia by exsanguinations and organs were rapidly removed. Testes were either frozen until isolation of mRNA and preparation for GAD activity measurements, or fixed in Bouin's solution overnight at 4°C and then embedded in paraffin.

Antibodies and antisera

For immunohistochemistry, immunocytochemistry and Western blot analyses we employed rabbit polyclonal antiserum against GAD, which recognizes both isoforms GAD65 and GAD67 (DPC Biermann, Bad Nauheim, Germany); rabbit polyclonal antiserum against VGAT (SySy Synaptic Systems GmbH, Göttingen, Germany); rabbit polyclonal antiserum against $GABA_A$-α1 (Alomone Labs Inc., Jerusalem, Israel), sheep polyclonal antiserum against $GABA_B$-R1 and $GABA_B$-R2 (gift from Graham Disney and Fiona Marshall, GlaxoWellcome R&D Inc., Stevenage, UK), mouse monoclonal antiserum against PCNA (Merck Biosciences, Schwalbach, Germany) and mouse monoclonal antiserum against β-Actin (Sigma, Deisenhofen, Germany).

Cell culture

TM3 cells are an established Leydig cell line. They derived from mouse Leydig cells [40,41] and were cultured in F12-DME medium (pH 7.2; Sigma, Deisenhofen, Germany) supplemented with 5×10^4 IU/l penicilline, 5×10^4 μg/l streptomycine, 5% horse serum (all from Biochrom AG, Berlin, Germany) and 2.5% fetal calf serum FCS Gold (PAA GmbH, Cölbe, Germany). AtT20 cells, a mouse adenohypophysial corticotroph tumor cell line [42,43], were cultured in F12-DME medium (pH 7.2; Sigma, Deisenhofen, Germany) supplemented with 2 mM L-glutamine (Sigma, Deisenhofen, Germany), 15% horse serum

Table 1: Sequences of oligonucleotide primers used for PCR amplification

Target	GenBank accession no.	Length (bp)	Primer sequence
GAD67	NM_008077	393	5'-CTTCTTCCGGATGGTCATCTC-3' 5'-ACGAGCAACATGCTATGGTCT-3'
GAD65	NM_008078	372	5'-GATATGGTTGGATTAGCAGC-3' 5'-CATTTTCCCTCTCTCATCAC-3'
VGAT	NM_009508	300	5'-CCTGTACGAGGAGAACGAAG-3' 5'-AGCAGACTGAACTTGGACAC-3'
GABA$_A$ α1	NM_010250	231	5'-CTACAGCAACCAGCTATACCC-3' 5'-GCTCTCTGTTTAAATACGTGG-3
GABA$_A$ α2	NM_008066	282	5'-AAGGCTCCGTCATGATACAG-3' 5'-ACTAACCCCTAATACAGGC-3'
GABA$_A$ α3	NM_008067	418	5'-GACTTGCTTGGTCATGTTGTTGGG-3' 5'-CAGAGGCCCTGGAGATGAAGAAGA-3'
GABA$_A$ β1	NM_008069	540	5'-ATGATGCATCTGCAGCCA-3' 5'-TGGAGTTCACGTCAGTCA-3'
GABA$_A$ β2	NM_008070	402	5'-GCATGTATGTCTGCAGGA-3' 5'-CTGACACCTACTTCCTGA-3'
GABA$_A$ β3	NM_008071	224	5'-AGCCAAGGCCAAGAATGATCG-3' 5'-TGCTTCTGTCTCCCATGTACC-3'
GABA$_A$ γ1	X55272	191	5'-TTTCTTACGTGACAGCAATGG-3' 5'-CATGGGAATGAGAGTGGATCC-3'
GABA$_A$ γ2	NM_008073	351	5'-GCAATGGATCTCTTTGTA-3' 5'-GTCCATTTTGGCAATGCG-3'
GABA$_A$ γ3	NM_008074	251	5'-TGTCGAAAGCCAACCATCAGG-3' 5'-GACTTGCACTCCTCATAGCAG-3'
GABA$_B$ R1	NM_019439	350	5'-ATGTGGTAACCATCCAGC-3' 5'-AGAAGATCGGCTACTACG-3'
GABA$_B$ R2	XM_194138	354	5'-CATCATCTTCTGTAGCAC-3' 5'-TCTGTGAAGTTGCCCAAG-3'

(Biochrom AG, Berlin, Germany) and 2.5% fetal calf serum FCS Gold (PAA GmbH, Cölbe, Germany). Both cell lines were kept at 37°C in a humidified atmosphere containing air and carbon dioxide (95%/5% vol/vol). In order to study proliferation and cellular PCNA content, TM3 cells were cultured for 24 h in serum-reduced medium (1% fetal calf serum, 2.5% horse serum). This treatment yields a synchronization of the cell cycle [40,44]. TM3 cells were incubated subsequently in the same serum-reduced medium with 10^{-5} M GABA, 10^{-5} M GABA$_A$ agonist isoguvacine, 10^{-5} M GABA$_B$ agonist baclofen, as well as combinations with 10^{-5} M GABA$_A$ antagonist bicuculline and 10^{-5} M GABA$_B$ antagonist phaclofen (BIOTREND GmbH, Köln, Germany) for 5, 10, 15, 30 min and for 24 h.

Cell proliferation studies

TM3 cells (5×10^3 cells per well) were plated on 96-well plates (Nunc, Wiesbaden, Germany) and incubated for 24 h in the presence or absence of GABA, isoguvacine, baclofen, bicuculline and phaclofen. One experiment included 32 replicate wells per treatment. As previously described [45,46], cell proliferation was determined by using the CellTiter 96 AQ$_{ueous}$ One Solution cell proliferation assay (Promega, Mannheim, Germany). The specificity and sensitivity of this method was previously evaluated in our lab by comparison with a [^{3}H]thymidine incorporation assay [45].

RNA preparation and RT-PCR

Isolation of RNA from rodent testes, as well as RT and PCR for GAD65/67, VGAT and GABA receptor subunits were performed as described previously [47]. Conditions of PCR amplification consisted of 30 or 35 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 60 s, followed by final extension for 5 min at 72°C). Oligonucleotide primers, as specified in Table 1, were synthesized according to published sequences. Verification of cDNAs was achieved by direct sequencing [47].

Immunohistochemistry

Testicular distribution of GAD, VGAT, PCNA and GABA$_{A/B}$ receptor subunits were examined in deparaffinized sections (5 μm) of Bouin's fixed testes of rats and mice using an Avidin-Biotin-Peroxidase (ABC) immunohistochemical method as described previously [48]. Specific antisera against GAD65/67 (dilution 1:500), VGAT (dilution 1:750), GABA$_A$-α1 (dilution 1:750), GABA$_B$-R1 (dilution 1:1.000–1:500), GABA$_B$-R2 (dilution 1:1000–1:500) and PCNA (dilution 1:1000–1:500) were employed. A biotin coupled polyclonal goat anti-rabbit antiserum (diluted 1:500; Jackson Inc., West Grove, USA), a biotin coupled goat anti-sheep antiserum (diluted 1:200; Dianova, Hamburg, Deutschland) or a biotin coupled goat anti-mouse antiserum (diluted 1:500; Jackson Inc., West Grove, USA) served as secondary antiserum. Diaminobenzidine (DAB) was used as a chromogen. Sections incubated with buffer alone, buffer containing mouse, sheep or rabbit non-immune serum, respectively, served as controls for all samples. The sections were examined with a Axiovert photomicroscope (Zeiss, Oberkochen, Germany).

Immunocytochemistry

TM3 cells were cultivated on glass cover slips (2×10^4 cells per cover slip) for 1 day. They were then fixed and handled as previously described [49]. For immunolocalization an antiserum recognizing GAD65/67 and an antiserum recognizing VGAT was carried out overnight at 4°C (diluted 1:1000 in 0.02 M potassium phosphate buffered saline containing 2% goat non-immune serum, pH 7.4). Immunoreactivity was visualized using a secondary polyclonal goat anti-rabbit antiserum (diluted 1:200; Dianova, Hamburg, Germany) labeled with fluorescein isothiocyanate (FITC). For control purposes either the specific antiserum was omitted or incubations with rabbit non-immune serum (dilution 1:10.000) were carried out instead. Sections were examined with a Axiovert microscope (Zeiss, Oberkochen, Germany), equipped with a FITC filter set.

Western blotting

Western blot analyses were performed with minor modifications as described previously [50]. In brief, TM3 cells and for control purposes tissue of mouse brain were lysated and homogenized in 62.5 mM Tris-HCL buffer (pH 6.8) containing 10% sucrose and 2% SDS by sonication, mercaptoethanol was added (10%), and the samples were heated (95°C for 5 min). Protein content was recorded [51] using a folin phenol quantitation method (DC protein assay, Bio-Rad GmbH, München, Germany). Then, 15 μg protein per lane was loaded on Tricine-SDS-polyacrylamide gels (12.5%), electrophoretically separated, and blotted onto nitrocellulose. Samples were probed with antiserum directed specifically against GAD65/67, PCNA and β-Actin (incubation overnight at 4°C, dilution 1:500). Immunoreactivity was detected using peroxidase labeled goat anti-rabbit antiserum (diluted: 1:5000; Jackson Inc., West Grove, USA) or peroxidase coupled goat anti-mouse antiserum (diluted: 1:5000; Jackson Inc., West Grove, USA) and enhanced chemiluminescence (Amersham Buchler, Braunschweig, Germany). Integrated optical density of Western blot reaction with antiserum directed against PCNA and β-Actin in TM3 cells was measured using Scion Image 4.0.2 (Scion Corporation, Frederick, USA) as previously described in detail [52].

GAD activity measurements

Determination of GAD activity by measuring the production of radiolabeled carbon dioxide (CO_2) from ^{14}C-glutamate was performed as described previously [7,53]. In brief, TM3 cells, AtT20 cells and rat tissue samples were homogenized in 60 mM potassium phosphate buffer (pH = 7.1), containing 0.5% Triton X-100, 1 mM 2-aminoethyl-isothiouronium bromide and 1 mM phenylmethanesulphonyl fluoride (Sigma, Deisenhofen, Germany), centrifuged, and the supernatants were used in the assay. The assay was performed in a total reaction volume of 60 μl, containing 20 μl of sample and 0.1 mM EDTA, 0.5% Triton X-100, 0.1 mM DTT, 0.05 mM pyridoxal phosphate, 9 mM L-glutamate, 3.3 μCi/ml ^{14}C-glutamate (Biotrend, Köln, Germany, specific activity: 50–60 mCi/mmol) and 60 mM potassium phosphate buffer. The reaction mix was incubated for 1 h at 37°C and then stopped by adding 100 μl of 10% trichloracetic acid per vial. The released CO_2 was absorbed on benzethonium hydroxide-drenched filter disks, and bound radioactivity was determined using a Tri-Carb 2100 liquid scintillation counter (Packard, Meriden, USA). The values obtained were normalized to protein content measured by DC protein assay (Bio-Rad GmbH, München, Germany) described above. Rat tissue samples that were heated to 95°C for 5 min served as negative controls.

Statistical analyses

Statistic analyses were performed using GraphPad Prism 3.02 (GraphPad Software, San Diego, USA). The results obtained in cell proliferation and GAD assay experiments were compared using one-way-analysis of variance ANOVA followed by Newmann-Keuls test. The results received in Western blot experiments were compared using one-way-analysis of variance ANOVA followed by Dunnett's test. Data shown are expressed as means+SEMs (standard error of the mean).

Results

A GABAergic system is present in postnatal rat testis: Active GAD, VGAT and GABA$_A$-α1 in postnatal rat Leydig cells

In postnatal rat testis immunohistochemical studies revealed that components of a local GABAergic system are present in interstitial cells (Figure 1). GAD65/67 and VGAT proteins were localized to the cytoplasm of interstitial cells. Because of their rounded morphology and clustered appearance these interstitial cells represent fetal Leydig cells. Protein of GABA$_A$-α1 was immunolocalized

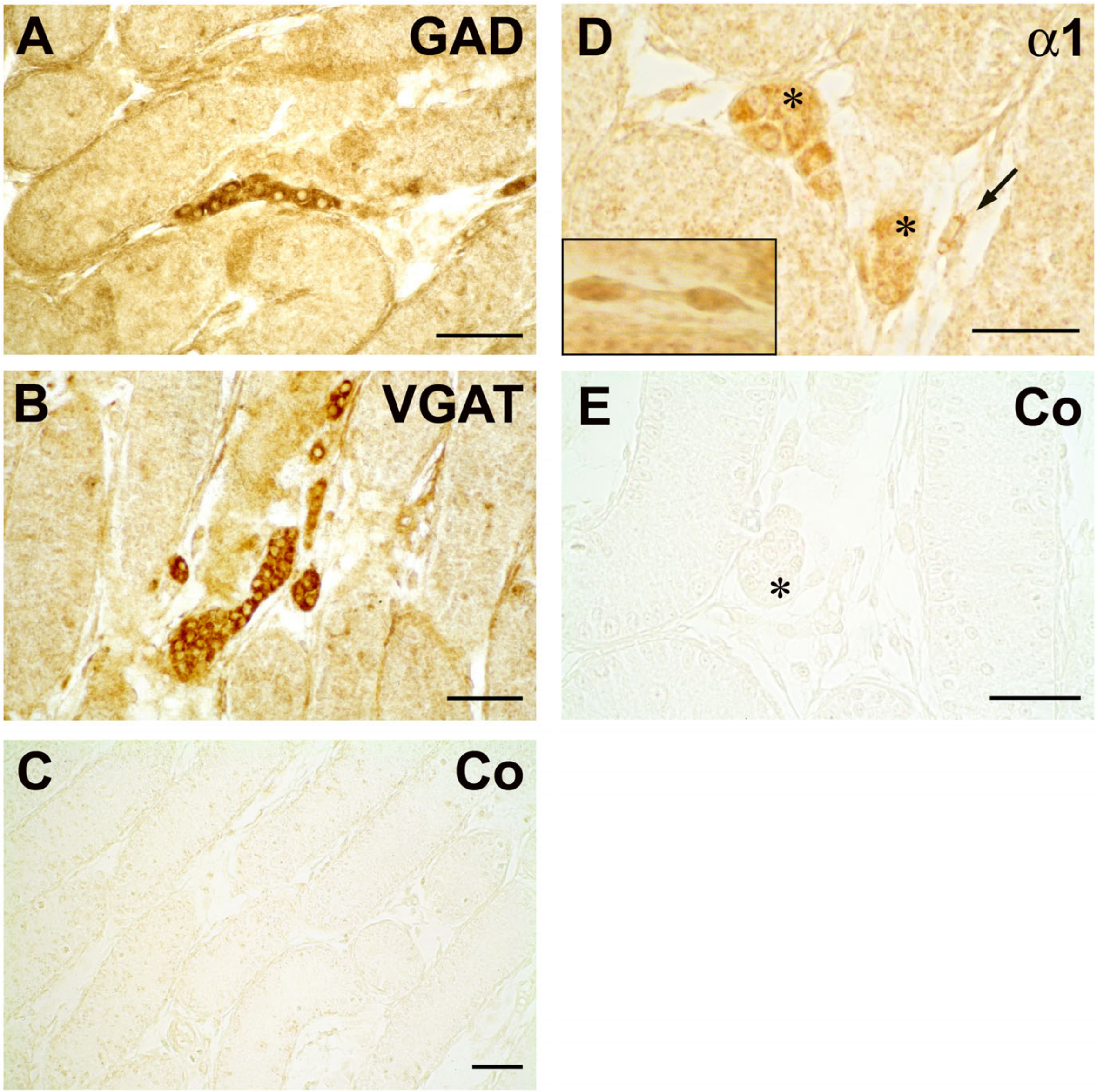

Figure 1

Leydig cells in postnatal rat testis possess GAD, VGAT and $GABA_A$-α1. Immunohistochemical localization of GAD, VGAT and $GABA_A$-α1 in the testis of five days old rats. Fetal Leydig cells with typical rounded morphology are located in clusters between the seminiferous tubules and are immunopositive for GAD (A) and VGAT (B). No reaction was observed in sections incubated with buffer (data not shown) or non-immune rabbit serum (C) instead of the primary antibody. The $GABA_A$ receptor subunit α1 (D) is also immunolocalized to clustered fetal Leydig cells (*). However other interstitial cells with a spindle-shaped appearance also exhibit specific immunoreactivity against $GABA_A$-α1 (D, →). The insert panel (D) also depicts magnified spindle-shaped interstitial cells of another section, which are immunopositive for $GABA_A$-α1. No reaction was observed in sections incubated with buffer (data not shown) or non-immune rabbit serum (E) instead of the primary antibody. Bars: 50 μm.

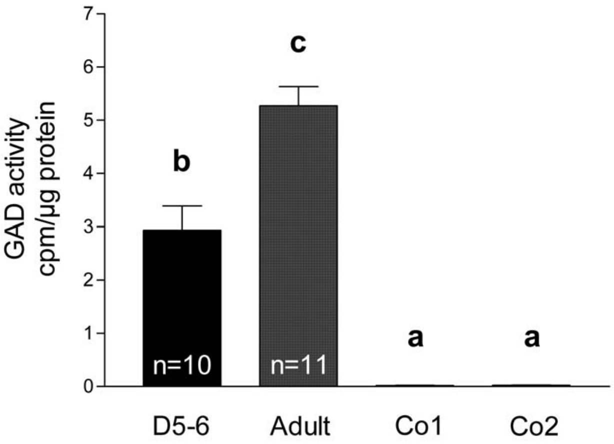

Figure 2
GAD is active in postnatal rat testis. GAD activity [cpm/µg protein] was measured in testicular tissue samples of 5–6 days old rats (D5-6, n = 10) and adult rats (Adult, n = 11). Tissue samples of adult rat testes (Co1, n = 6) and adult rat cerebella (Co2, n = 6) heated to 95°C for 5 min served as negative controls. GAD activity in adult testes is higher than GAD activity in postnatal testes. Columns with different superscripts are significantly (ANOVA/Newmann-Keuls, $p < 0.001$) different from each other and represent means+SEMs.

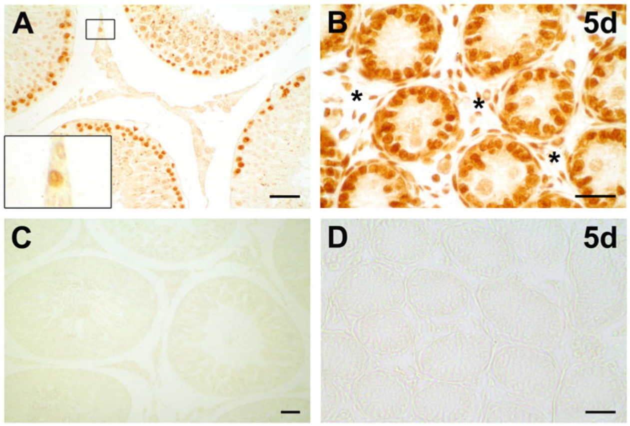

Figure 3
PCNA in postnatal and adult rat testis. Immunohistochemical testicular localization of the proliferation marker PCNA (proliferating cell nuclear antigen) revealed that germ cells and Sertoli cells are immunopositive for PCNA, as seen in adult rat testis (A) and five days old (5d) rat testis (B). In interstitial spaces of adult testis (A) we sporadically found immunopositive cells (insert panel, A). In postnatal testis (B) abundant immunoreactive cells (*) were identified in the interstitium. No reaction was observed in sections incubated with buffer or non-immune serum (C, D) instead of the primary antibody. Bars: 25 µm.

not only to clustered fetal Leydig cells, but also to spindle-shaped interstitial cells. When antisera against GABA$_B$-R1 and GABA$_B$-R2 were employed, specific immunoreactive signals were absent. All control panels probed with buffer alone or non-immune rabbit serum were negative.

Evidence for enzymatically active GAD65/67 protein in postnatal rat testes was provided by measurements of ^{14}C1-glutamic acid decarboxylation (Figure 2). Tissue of adult rat testes, which is known to possess GAD activity [7], served as positive control. GAD activity in postnatal (n = 10) rat testes was clearly detected (2.94 ± 0.45 cpm/µg protein), but was significantly lower than in adult (n = 11) rat testes (5.27 ± 0.36 cpm/µg protein; ANOVA/Newmann-Keuls, $p < 0.001$). Both values are significantly higher (ANOVA/Newmann-Keuls, $p < 0.001$) from background and tissues, in which GAD was heat-inactivated (adult rat testes (n = 6) and adult rat cerebella (n = 6) heated to 95°C for 5 min).

Proliferation marker PCNA is localized in interstitial cells of postnatal rodent testis

To identify proliferating cells in adult and postnatal rat testes we probed testicular tissue sections of rats (Figure 3) and mice (data not shown) with an antiserum against the proliferation marker PCNA. Specific immunoreactions were observed inside the seminiferous tubules on germ cells and in Sertoli cells, as well as in the cells of the interstitial compartment of all samples examined. In contrast, in adult rat testes we only occasionally found interstitial cells to be immunopositive for PCNA. In controls performed with buffer alone or buffer containing non-immune mouse serum, respectively, specific immunoreactivity was absent.

The GABAergic system is also present in postnatal mouse testis: GAD67, VGAT and several GABA$_A$ receptor subunits in postnatal mouse testis

RT-PCR studies identified mRNAs of VGAT and GAD67, but not of GAD65 (data not shown). Furthermore, mRNAs of the GABA$_A$ receptor subunits α2, β1, β2, β3 and γ3 were readily detected (Table 2). The mRNAs of the GABA$_A$ receptor subunits α1, α3, γ1, γ2 and of the GABA$_B$ receptor subunits R1 and R2 were not found in postnatal mouse testis in several RT-PCR experiments. Immunohistochemical experiments (using GAD and VGAT antisera) yielded results similar to the ones obtained in rat (data not shown).

Table 2: Distribution of GABA$_A$ receptor subunits in postnatal mouse testis (D5) and in TM3 cells revealed by RT-PCR

	GABA$_A$ receptor subunits								
	α1	α2	α3	β1	β2	β3	γ1	γ2	γ3
D5	-	+	-	+	+	+	-	-	+
TM3	+	+	-	+	-	+	+	-	-

The RT-PCR results (+) in postnatal mouse testis (D5) and in TM3 cells were confirmed by sequencing; (-) indicates that the expected mRNA was not detected in several RT-PCR experiments.

TM3 Leydig cells possess active GAD67, VGAT and GABA$_A$ receptor subunits α1, α2 β1, β3 and γ1

To examine whether mouse-derived TM3 cells may serve as model for proliferating Leydig cells, we first determined whether TM3 cells are able to produce GABA and possess GABA receptors. Specific immunocytochemical staining against GAD65/67 and VGAT protein was observed in TM3 cells (Figure 4). Specific immunoreactivity was absent in controls performed with buffer alone or buffer containing non-immune rabbit serum, respectively. RT-PCR and Western blot experiments confirmed these results and revealed GAD67, but not GAD65, in TM3 cells (Figure 5). GAD67 protein in TM3 cells was found to be enzymatically active (Figure 6) with an assayed GAD activity of 3.25 ± 0.24 cpm/μg protein (n = 11). TM3 cells (n = 6) and rat testicular tissue (n = 6), both heated to 95°C for 5 min, served as negative controls. GAD activity of TM3 cells was significantly (ANOVA/Newmann-Keuls, $p < 0.001$) higher than GAD activity (0.46 ± 0.05 cpm/μg protein) of AtT20 cells (n = 9), another mouse cell line. GAD activities of AtT20 cells and of negative controls (inactivated by boiling) were not significantly different from each other (ANOVA/Newmann-Keuls, $p > 0.05$).

Furthermore, mRNAs of the GABA$_A$ receptor subunits α1, α2, β1, β3 and γ1 were readily detected in TM3 cells (Table 2). In contrast the mRNAs of the GABA$_A$ receptor subunits α3, β2, γ2 and γ3, as well as the mRNAs of the GABA$_B$ receptor subunits R1 and R2 were not found in TM3 cells in several RT-PCR experiments.

GABA and GABA$_A$ agonist isoguvacine increase cellular content of PCNA in TM3 cells

Levels of the proliferation marker PCNA was determined using Western blotting after stimulation with GABA or GABA receptor agonists/antagonists in TM3 cells (Figure 7). TM3 cells were incubated 0, 5, 10, 15, 30 min with GABA, GABA+bicuculline, isoguvacine, isoguvacine+bicuculline, baclofen and baclofen+phaclofen, respectively, and PCNA content was semiquantitatively determined by Western blotting. Signals were normalized and thus corrected for minor loading differences with the help of the results obtained for β-Actin (n = 5 experiments per treatment). Stimulation with GABA or GABA$_a$ agonist isoguvacine lasting for 15 min significantly (ANOVA/Dunnett's, $p < 0.001$) increased PCNA content in TM3 cells compared to untreated samples (0 min stimulation). This effect was blocked by co-incubation with GABA$_a$ antagonist bicuculline. Stimulation with GABA$_B$ agonists or antagonists (baclofen, and baclofen+phaclofen) did not alter PCNA content in TM3 cells (data not shown).

GABA induced TM3 cell proliferation is mediated by GABA$_A$ receptor

In order to investigate whether GABA$_A$ receptor activation is not only able to increase PCNA content in TM3 cells, but indeed can stimulate TM3 cell proliferation, we performed proliferation assays (Figure 8). TM3 cells were incubated for 24 h with GABA (n = 22), isoguvacine (n = 21), baclofen (n = 25), GABA+bicuculline (n = 19) and isoguvacine+bicuculline (n = 13). GABA significantly (ANOVA/Newmann-Keuls, $p < 0.05$) increased TM3 cell proliferation up to 124.3 ± 4.4% compared to untreated controls (n = 36; 100%). Stimulation with isoguvacine also significantly (ANOVA/Newmann-Keuls, $p < 0.05$) increased cell proliferation up to 120.7 ± 4.1%, but baclofen treatment did not result in significant alteration in cell proliferation. Further evidence for involvement of GABA$_A$ receptors was provided by blocking of the proliferative effects of GABA and isoguvacine by GABA$_A$ antagonist bicuculline (ANOVA/Newmann-Keuls, $p < 0.05$).

Discussion

The present study shows that crucial components of a GABAergic system are present in the endocrine compartment of postnatal rodent testis and that GABA stimulates proliferation of TM3 Leydig cells via GABA$_A$ receptors. These results suggest that GABA may regulate cell proliferation of fetal Leydig cells and/or mesenchymal precursors of the adult Leydig cell lineage in an auto-/paracrine manner. We therefore suggest that GABA may contribute to the morphogenesis of the testis.

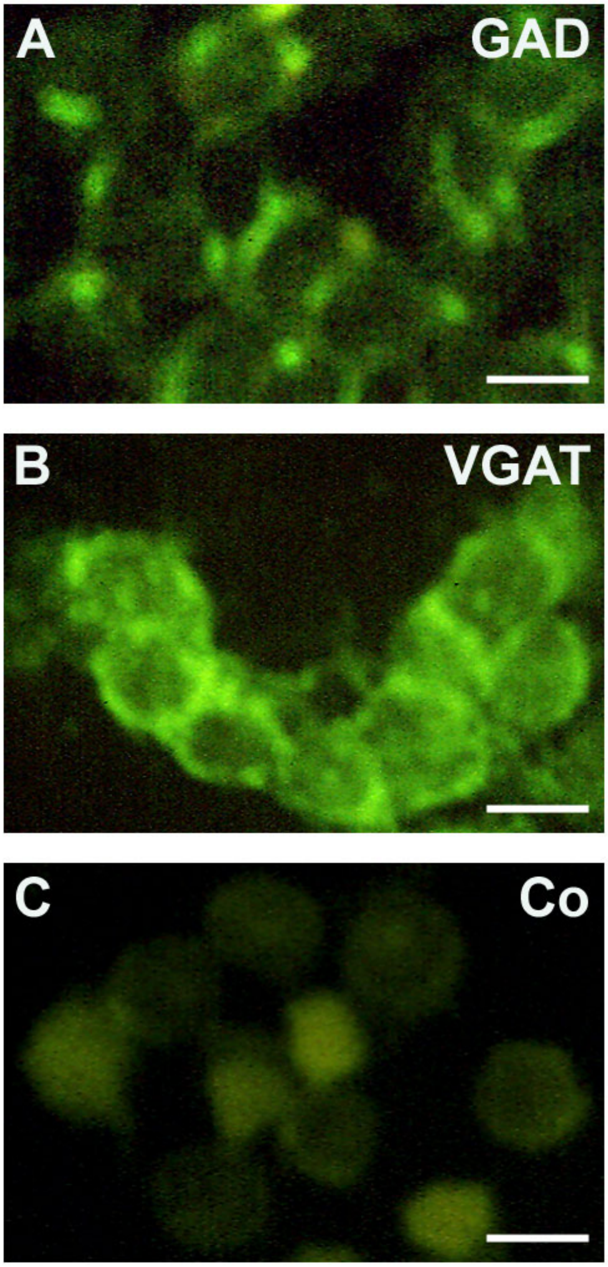

Figure 4
Immmunocytochemical evidence for the presence of GAD and VGAT in TM3 cells. A cytoplasmatic staining pattern for GAD (glutamate decarboxylase) and VGAT (vesicular GABA transporter) was observed in TM3 cells (A, B). Controls included using non-immune serum (C) and omission of primary antiserum. Bars: 15 μm.

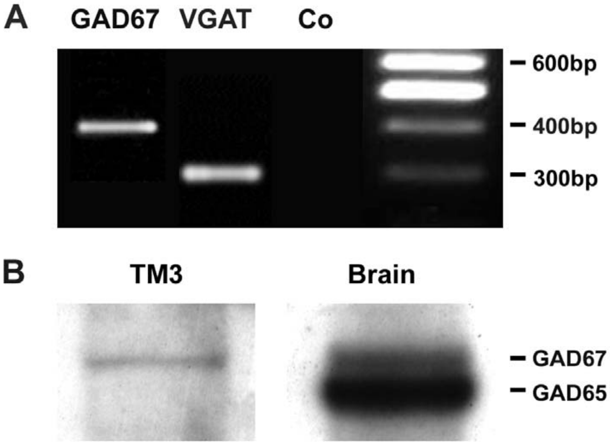

Figure 5
RT-PCR and Western blot results: TM3 cells express GAD67 and VGAT. RT-PCR experiments (A) revealed that TM3 cells possess mRNA for GAD67, but lack GAD65 (data not shown), as well as VGAT. Expected product sizes were 399 bp for GAD67 and 302 bp for VGAT. Western blot analysis (B) revealed the presence of GAD67 in TM3 cells. Protein probes from mouse brain served as positive control and depict immunoreactivity for both GAD isoforms.

Previously we and others demonstrated first details of a local GABAergic system in the endocrine compartment of adult rodent and human testis [7,54]. Adult Leydig cells possess enzymatically active GAD, VGAT and several GABA$_A$ and GABA$_B$ receptor subunits. Both isoforms GAD65 and GAD67 were present in rats and mice [7]. The functional significance of a testicular GABAergic system is not well known, but auto-/paracrine modulation of testosterone production in Leydig cells is a possibility suggested by studies describing stimulating effects of GABA on testosterone production in rats [8,9]. Since hormonal influences clearly govern steroid production of the adult testis, the modulatory effect of GABA on testosterone may however not be the main effect of GABA.

We rather speculated that GABA may exert trophic effects in the testis. This assumption was based on the trophic action of GABA via GABA$_A$ receptors in the developing brain. We focused in this study therefore on the postnatal testis, which bears proliferating cells including fetal Leydig cells and cells of the adult Leydig cell lineage.

It is widely accepted that two distinct Leydig cell populations are present during the first postnatal week in mouse and rat testis [33,34,36-39], namely steroidogenic fetal Leydig cells and mesenchymal precursors of adult Leydig cells. The first mentioned form conspicuous clusters in the interstitium [31,34,55]. Although fetal Leydig cells

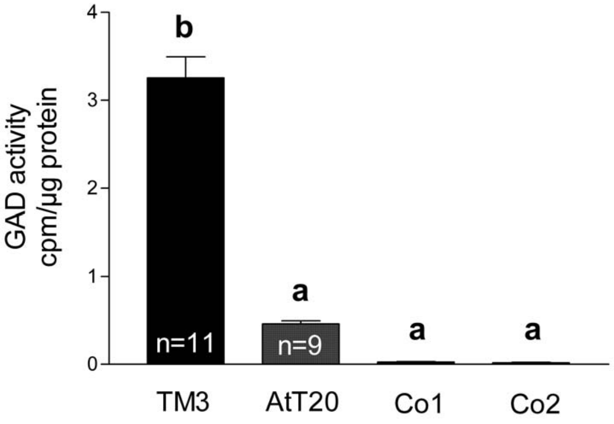

Figure 6
GAD is active in TM3 cells. GAD activity [cpm/µg protein] was measured in TM3 cells (n = 11 batches of cells) and in AtT20 cells (n = 9 batches of cells), another endocrine mouse cell line used for control purposes. TM3 cells (Co1, n = 6 batches of cells) and testicular samples of adult rats (Co2, n = 6), both heated to 95°C for 5 min, served as additional negative controls. Testicular GAD activity in TM3 cells is higher than GAD activity in AtT20 cells or in controls. Columns with different superscripts are significantly (ANOVA/ Newmann-Keuls, $p < 0.001$) different from each other and represent means+SEMs.

represent a differentiated cell population, there is evidence for a moderate increase in the number of these cells during the first two weeks of postnatal development [31,39]. In contrast, non-steroidogenic mesenchymal precursor cells of the adult Leydig cell lineage are located primarily in peritubular regions and proliferate strongly. They differentiate during the second postnatal week into progenitor cells and from the end of the third week on into newly formed, immature and then into fully functional mature adult Leydig cells [33,34,36-38].

Our immunohistochemical findings indeed evidenced dramatic proliferative events in the postnatal testis. Interstitial and peritubular cells in the testis of five days old rats expressed the proliferation marker PCNA, which was used in testicular tissues before [56-58]. Among these cells are likely connective tissue cells and endothelial cells [59-61], but also fetal Leydig cells and mesenchymal precursors of adult Leydig cells, as judged by their typical location and morphology. Since the latter are undifferentiated in nature [34,36-38], we could not use specific markers to distinguish them from other cell types.

Our study links Leydig cells proliferation and local testicular GABA synthesis. This is based first on the fact that we identified GABA synthesis and $GABA_A$ receptors in the postnatal testis of rodents, and second on the proliferative action of GABA and $GABA_A$ agonists in TM3 Leydig cells.

We identified only fetal Leydig cells, characterized by their rounded morphology and clustered appearance in the testicular interstitium, to possess GAD67 and VGAT. In contrast to adult testis, GAD65 was not detected. GAD67 was, however, found to be enzymatically active in rat testicular tissue of the same developmental stage. These two results together allow the conclusion that only fetal Leydig cells possess the pivotal molecules to synthesize and store GABA.

The present investigation provides insights into the possible targets of testicular GABA. As evidenced by RT-PCR studies, several $GABA_A$ receptor subunits are expressed in the postnatal testis. $GABA_B$ receptors were not found in postnatal testis, a result in contrast to our previous study in the adult rodent testis [7]. Immunolocalization of $GABA_A$ receptor subunits was hampered, due to availability of suitable antisera, but localization of $GABA_A$-α1 revealed presence on rat fetal Leydig cells, but also on spindle-shaped interstitial cells. At least some of the last mentioned cells are very likely to represent mesenchymal precursor cells of the adult Leydig cell lineage. Thus according to the immunolocalization of $GABA_A$-α1, both fetal Leydig cells and precursors of adult Leydig cells, are possible targets for GABA in the postnatal testis.

The number of fetal Leydig cells increases moderately in rodents during the first two weeks of postnatal development [31,39] and it is possible that GABA may mediate this effect. Another possibility is that GABA may modulate cell proliferation of mesenchymal precursor cells of adult Leydig cells or other $GABA_A$ receptor bearing testicular cell types. Based on our results in the present study, it is possible that GABA might even be a start signal leading to proliferation and differentiation of mesenchymal precursors of adult Leydig cells. Interestingly, this signal is as yet unknown [34,37,38]. Thyroid hormone may be involved, but participation of LH and androgens in the initiation of adult Leydig cell development was ruled out [37,39,62-66].

Clearly, in-vivo evidence for such a crucial role of testicular GABA is as yet missing, but unequivocal evidence for a proliferative action of GABA via $GABA_A$ receptors was provided by cell culture experiments using TM3 Leydig cells, which possess $GABA_A$ receptor subunits. Involvement of $GABA_A$ receptors was suggested by the use of the pharmacologically well defined $GABA_A$ agonist isoguvacine and by the use of the $GABA_A$ antagonist

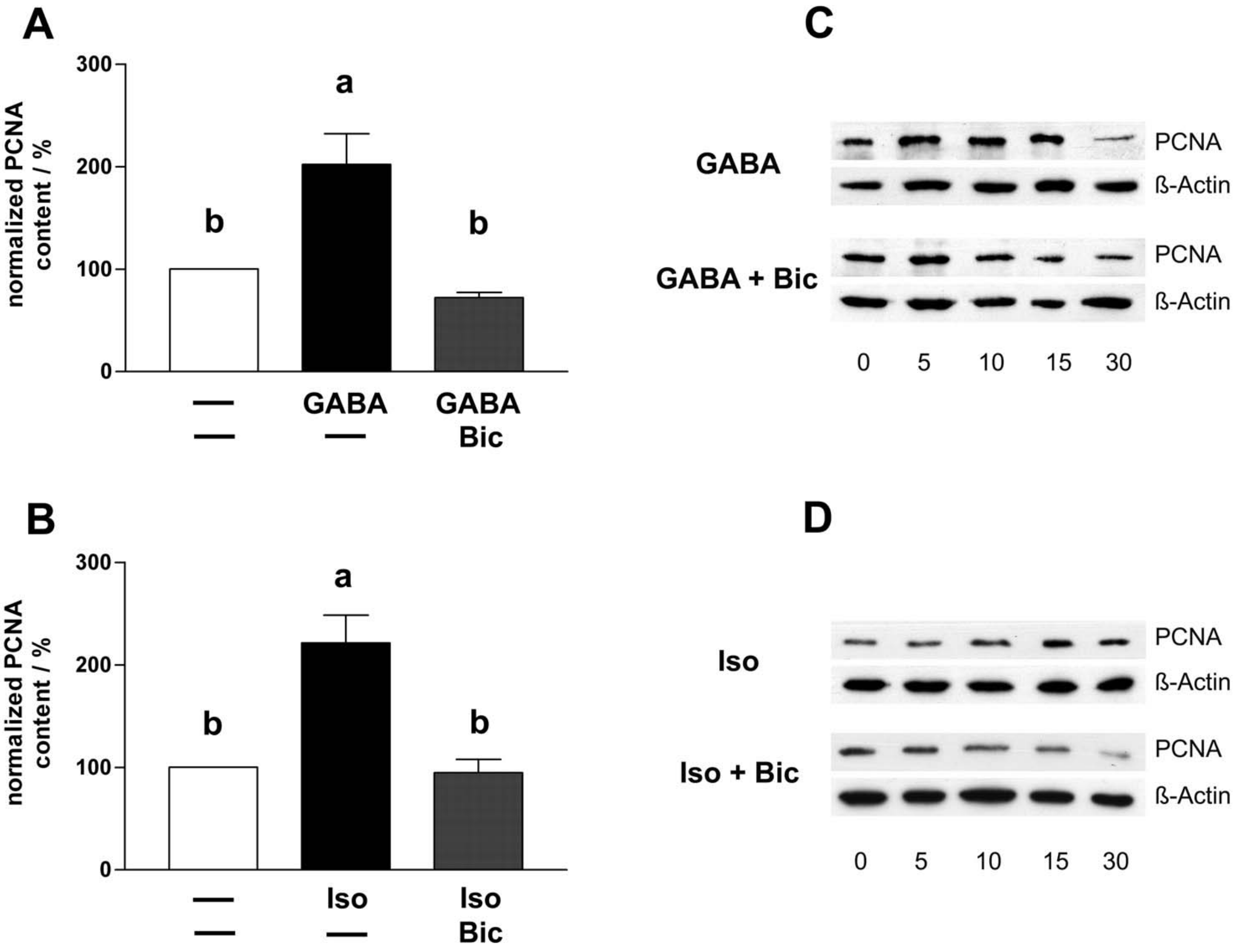

Figure 7
PCNA content in TM3 cells is increased by GABA and $GABA_A$ agonist isoguvacine. Using Western blot analyses we examined PCNA content of TM3 cells after 0, 5, 10, 15, 30 min stimulation with GABA, GABA+bicuculline, isoguvacine and isoguvacine+bicuculline, respectively. The PCNA content of TM3 cells after 15 min stimulation with GABA (A) is significantly higher compared to untreated TM3 cells or compared to TM3 cells stimulated for 15 min with GABA+bicuculline. After 15 min stimulation with isoguvacine (B) the PCNA content of TM3 cells is also significantly higher compared to untreated TM3 cells or compared to TM3 cells stimulated for 15 min with isoguvacine+bicuculline. Data represent means+SEMs of n = 5 independent experiments and were normalized to β-Actin levels. Columns with different superscripts are significantly ($p < 0.001$, ANOVA/Dunnett's test) different from each other. Figure 7C and 7D depict representative Western blot experiments, respectively.

bicuculline. Interestingly, this signaling pathway is in analogy to studies in the developing brain, where GABA also induces cell proliferation of neuronal progenitors and other neuronal cell types via activation of $GABA_A$ receptors [10-13].

In summary, a GABAergic system exists already in postnatal rodent testis and differs from the one in adult testis, since one of the two GAD isoforms as well as $GABA_B$ receptor subunits are missing. Nevertheless, it appears functional and our results suggest that GABA has similar roles in the developing brain and in the developing testis, namely to act as a trophic factor affecting the morphogenesis of crucial cells in these two organs.

Authors' contributions

CG carried out most of the experiments, participated in the study design, performed statistical analyses and drafted the manuscript. RFGD carried out part of RT-PCR, Western blotting and immunocytochemistry. AT and AK

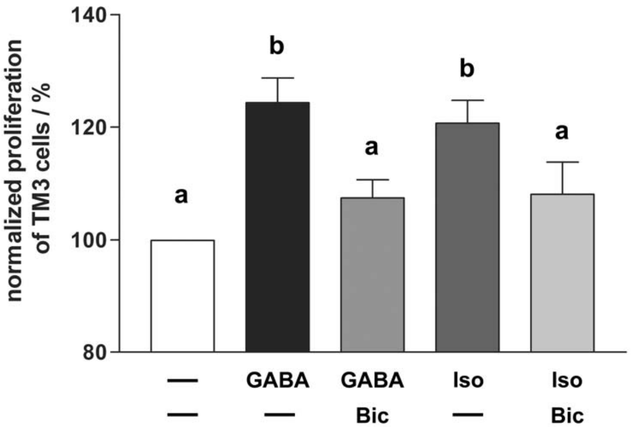

Figure 8
GABA and $GABA_A$ agonist isoguvacine stimulate TM3 cell proliferation. Summary graph of 13–36 independent experiments (means+SEMs). TM3 cells were incubated for 24 h with GABA (n = 22), GABA+bicuculline (n = 19), isoguvacine (n = 21) and isoguvacine+bicuculline (n = 13). Both GABA and isoguvacine stimulated TM3 cell proliferation, whereas addition of bicuculline blocked these effects. All results were normalized to proliferation of unstimulated TM3 cells (n = 36), serving as control panel. Columns with different superscripts are significantly different from each other (ANOVA/Newmann-Keuls, $p < 0.05$).

provided technical assistance. AM conceived of the study, and participated in its design, coordination and writing of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Marlies Rauchfuss and Andreas Mauermayer for their expert technical assistance and Lars Kunz and Martin Albrecht for helpful discussions. We thank Prof. Ricardo S. Calandra and Dr. Silvia Gonzalez-Calvar for providing some of the mouse samples. This study was supported by DFG-Graduiertenkolleg 333 "Biology of human diseases".

References

1. Mayerhofer A, Hohne-Zell B, Gamel-Didelon K, Jung H, Redecker P, Grube D, Urbanski HF, Gasnier B, Fritschy JM, Gratzl M: **Gamma-aminobutyric acid (GABA): a para- and/or autocrine hormone in the pituitary.** *FASEB J* 2001, **15:**1089-1091.
2. Gamel-Didelon K, Corsi C, Pepeu G, Jung H, Gratzl M, Mayerhofer A: **An autocrine role for pituitary GABA: activation of GABA-B receptors and regulation of growth hormone levels.** *Neuroendocrinology* 2002, **76:**170-177.
3. Gamel-Didelon K, Kunz L, Fohr KJ, Gratzl M, Mayerhofer A: **Molecular and physiological evidence for functional gamma-aminobutyric acid (GABA)-C receptors in growth hormone-secreting cells.** *J Biol Chem* 2003, **278:**20192-20195.
4. Rorsman P, Berggren PO, Bokvist K, Ericson H, Mohler H, Ostenson CG, Smith PA: **Glucose-inhibition of glucagon secretion involves activation of GABAA-receptor chloride channels.** *Nature* 1989, **341:**233-236.
5. Gilon P, Bertrand G, Loubatieres-Mariani MM, Remacle C, Henquin JC: **The influence of gamma-aminobutyric acid on hormone release by the mouse and rat endocrine pancreas.** *Endocrinology* 1991, **129:**2521-2529.
6. Satin LS, Kinard TA: **Neurotransmitters and their receptors in the islets of Langerhans of the pancreas: what messages do acetylcholine, glutamate, and GABA transmit?** *Endocrine* 1998, **8:**213-223.
7. Geigerseder C, Doepner R, Thalhammer A, Frungieri MB, Gamel-Didelon K, Calandra RS, Kohn FM, Mayerhofer A: **Evidence for a GABAergic system in rodent and human testis: local GABA production and GABA receptors.** *Neuroendocrinology* 2003, **77:**314-323.
8. Ritta MN, Calandra RS: **Occurrence of GABA in rat testis and its effect on androgen production.** *Adv Biochem Psychopharmacol* 1986, **42:**291-297.
9. Ritta MN, Campos MB, Calandra RS: **Effect of GABA and benzodiazepines on testicular androgen production.** *Life Sci* 1987, **40:**791-798.
10. Ben Ari Y, Tseeb V, Raggozzino D, Khazipov R, Gaiarsa JL: **gamma-Aminobutyric acid (GABA): a fast excitatory transmitter which may regulate the development of hippocampal neurones in early postnatal life.** *Prog Brain Res* 1994, **102:**261-273.
11. Lauder JM, Liu J, Devaud L, Morrow AL: **GABA as a trophic factor for developing monoamine neurons.** *Perspect Dev Neurobiol* 1998, **5:**247-259.
12. Kriegstein AR, Owens DF: **GABA may act as a self-limiting trophic factor at developing synapses.** *Sci STKE* 2001, **95:**E1-.
13. Owens DF, Kriegstein AR: **Is there more to GABA than synaptic inhibition?** *Nat Rev Neurosci* 2002, **3:**715-727.
14. Ben Yaakov G, Golan H: **Cell proliferation in response to GABA in postnatal hippocampal slice culture.** *Int J Dev Neurosci* 2003, **21:**153-157.
15. Nguyen L, Rigo JM, Rocher V, Belachew S, Malgrange B, Rogister B, Leprince P, Moonen G: **Neurotransmitters as early signals for central nervous system development.** *Cell Tissue Res* 2001, **305:**187-202.
16. Fiszman ML, Borodinsky LN, Neale JH: **GABA induces proliferation of immature cerebellar granule cells grown in vitro.** *Brain Res Dev Brain Res* 1999, **115:**1-8.
17. Haydar TF, Wang F, Schwartz ML, Rakic P: **Differential modulation of proliferation in the neocortical ventricular and subventricular zones.** *J Neurosci* 2000, **20:**5764-5774.
18. Stewart RR, Hoge GJ, Zigova T, Luskin MB: **Neural progenitor cells of the neonatal rat anterior subventricular zone express functional GABA(A) receptors.** *J Neurobiol* 2002, **50:**305-322.
19. Borodinsky LN, O'Leary D, Neale JH, Vicini S, Coso OA, Fiszman ML: **GABA-induced neurite outgrowth of cerebellar granule cells is mediated by GABA(A) receptor activation, calcium influx and CaMKII and erk1/2 pathways.** *J Neurochem* 2003, **84:**1411-1420.
20. Kim MO, Li S, Park MS, Hornung JP: **Early fetal expression of GABA(B1) and GABA(B2) receptor mRNAs on the development of the rat central nervous system.** *Brain Res Dev Brain Res* 2003, **143:**47-55.
21. Wang DD, Krueger DD, Bordey A: **GABA depolarizes neuronal progenitors of the postnatal subventricular zone via GABAA receptor activation.** *J Physiol* 2003, **550:**785-800.
22. Fueshko SM, Key S, Wray S: **GABA inhibits migration of luteinizing hormone-releasing hormone neurons in embryonic olfactory explants.** *J Neurosci* 1998, **18:**2560-2569.
23. Behar TN, Schaffner AE, Scott CA, O'Connell C, Barker JL: **Differential response of cortical plate and ventricular zone cells to GABA as a migration stimulus.** *J Neurosci* 1998, **18:**6378-6387.
24. Behar TN, Schaffner AE, Scott CA, Greene CL, Barker JL: **GABA receptor antagonists modulate postmitotic cell migration in slice cultures of embryonic rat cortex.** *Cereb Cortex* 2000, **10:**899-909.
25. Bormann J: **The 'ABC' of GABA receptors.** *Trends Pharmacol Sci* 2000, **21:**16-19.
26. Liu J, Morrow AL, Devaud L, Grayson DR, Lauder JM: **GABAA receptors mediate trophic effects of GABA on embryonic brainstem monoamine neurons in vitro.** *J Neurosci* 1997, **17:**2420-2428.
27. Maric D, Liu QY, Maric I, Chaudry S, Chang YH, Smith SV, Sieghart W, Fritschy JM, Barker JL: **GABA expression dominates neuronal lineage progression in the embryonic rat neocortex and**

facilitates neurite outgrowth via GABA(A) autoreceptor/Cl- channels. *J Neurosci* 2001, **21:**2343-2360.

28. Amat P, Paniagua R, Nistal M, Martin A: **Mitosis in adult human Leydig cells.** *Cell Tissue Res* 1986, **243:**219-221.
29. Mendis-Handagama SM: **Mitosis in normal adult guinea pig Leydig cells.** *J Androl* 1991, **12:**240-243.
30. Russell LD, de Franca LR, Hess R, Cooke P: **Characteristics of mitotic cells in developing and adult testes with observations on cell lineages.** *Tissue Cell* 1995, **27:**105-128.
31. Kuopio T, Tapanainen J, Pelliniemi LJ, Huhtaniemi I: **Developmental stages of fetal-type Leydig cells in prepubertal rats.** *Development* 1989, **107:**213-220.
32. Lejeune H, Habert R, Saez JM: **Origin, proliferation and differentiation of Leydig cells.** *J Mol Endocrinol* 1998, **20:**1-25.
33. Baker PJ, O'Shaughnessy PJ: **Role of gonadotrophins in regulating numbers of Leydig and Sertoli cells during fetal and postnatal development in mice.** *Reproduction* 2001, **122:**227-234.
34. Habert R, Lejeune H, Saez JM: **Origin, differentiation and regulation of fetal and adult Leydig cells.** *Mol Cell Endocrinol* 2001, **179:**47-74.
35. Rouiller-Fabre V, Levacher C, Pairault C, Racine C, Moreau E, Olaso R, Livera G, Migrenne S, Delbes G, Habert R: **Development of the foetal and neonatal testis.** *Andrologia* 2003, **35:**79-83.
36. Benton L, Shan LX, Hardy MP: **Differentiation of adult Leydig cells.** *J Steroid Biochem Mol Biol* 1995, **53:**61-68.
37. Ariyaratne HB, Mendis-Handagama SM, Buchanan HD, Ian MJ: **Studies on the onset of Leydig precursor cell differentiation in the prepubertal rat testis.** *Biol Reprod* 2000, **63:**165-171.
38. Mendis-Handagama SM, Ariyaratne HB: **Differentiation of the adult Leydig cell population in the postnatal testis.** *Biol Reprod* 2001, **65:**660-671.
39. Ariyaratne HB, Mendis-Handagama SM: **Changes in the testis interstitium of Sprague Dawley rats from birth to sexual maturity.** *Biol Reprod* 2000, **62:**680-690.
40. Mather JP: **Establishment and characterization of two distinct mouse testicular epithelial cell lines.** *Biol Reprod* 1980, **23:**243-252.
41. Lee W, Mason AJ, Schwall R, Szonyi E, Mather JP: **Secretion of activin by interstitial cells in the testis.** *Science* 1989, **243:**396-398.
42. Orth DN, Nicholson WE, Mitchell WM, Island DP, Shapiro M, Byyny RL: **ACTH and MSH production by a single cloned mouse pituitary tumor cell line.** *Endocrinology* 1973, **92:**385-393.
43. Eipper BA, Mains RE: **High molecular weight forms of adrenocorticotropic hormone in the mouse pituitary and in a mouse pituitary tumor cell line.** *Biochemistry* 1975, **14:**3836-3844.
44. Mather JP, Zhuang LZ, Perez-Infante V, Phillips DM: **Culture of testicular cells in hormone-supplemented serum-free medium.** *Ann N Y Acad Sci* 1982, **383:**44-68.
45. Sommersberg B, Bulling A, Salzer U, Frohlich U, Garfield RE, Amsterdam A, Mayerhofer A: **Gap junction communication and connexin 43 gene expression in a rat granulosa cell line: regulation by follicle-stimulating hormone.** *Biol Reprod* 2000, **63:**1661-1668.
46. Frungieri MB, Weidinger S, Meineke V, Kohn FM, Mayerhofer A: **Proliferative action of mast-cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPARgamma : Possible relevance to human fibrotic disorders.** *Proc Natl Acad Sci U S A* 2002, **99:**15072-15077.
47. Fritz S, Fohr KJ, Boddien S, Berg U, Brucker C, Mayerhofer A: **Functional and molecular characterization of a muscarinic receptor type and evidence for expression of choline-acetyltransferase and vesicular acetylcholine transporter in human granulosa-luteal cells.** *J Clin Endocrinol Metab* 1999, **84:**1744-1750.
48. Mayerhofer A, Frungieri MB, Fritz S, Bulling A, Jessberger B, Vogt HJ: **Evidence for catecholaminergic, neuronlike cells in the adult human testis: changes associated with testicular pathologies.** *J Androl* 1999, **20:**341-347.
49. Mayerhofer A, Danilchik M, Pau KY, Lara HE, Russell LD, Ojeda SR: **Testis of prepubertal rhesus monkeys receives a dual catecholaminergic input provided by the extrinsic innervation and an intragonadal source of catecholamines.** *Biol Reprod* 1996, **55:**509-518.
50. Hohne-Zell B, Gratzl M: **Adrenal chromaffin cells contain functionally different SNAP-25 monomers and SNAP-25/syntaxin heterodimers.** *FEBS Lett* 1996, **394:**109-116.
51. Peterson GL: **Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall.** *Anal Biochem* 1979, **100:**201-220.
52. Grosse J, Bulling A, Brucker C, Berg U, Amsterdam A, Mayerhofer A, Gratzl M: **Synaptosome-associated protein of 25 kilodaltons in oocytes and steroid-producing cells of rat and human ovary: molecular analysis and regulation by gonadotropins.** *Biol Reprod* 2000, **63:**643-650.
53. Krieger NR, Heller JS: **Localization of glutamic acid decarboxylase within laminae of the rat olfactory tubercle.** *J Neurochem* 1979, **33:**299-302.
54. Frungieri MB, Gonzalez-Calvar SI, Chandrashekar V, Rao JN, Bartke A, Calandra RS: **Testicular gamma-aminobutyric acid and circulating androgens in Syrian and Djungarian hamsters during sexual development.** *Int J Androl* 1996, **19:**164-170.
55. Huhtaniemi I, Pelliniemi LJ: **Fetal Leydig cells: cellular origin, morphology, life span, and special functional features.** *Proc Soc Exp Biol Med* 1992, **201:**125-140.
56. Sriraman V, Rao VS, Sairam MR, Rao AJ: **Effect of deprival of LH on Leydig cell proliferation: involvement of PCNA, cyclin D3 and IGF-1.** *Mol Cell Endocrinol* 2000, **162:**113-120.
57. Chieffi P, Franco R, Fulgione D, Staibano S: **PCNA in the testis of the frog, Rana esculenta: a molecular marker of the mitotic testicular epithelium proliferation.** *Gen Comp Endocrinol* 2000, **119:**11-16.
58. Liang JH, Sankai T, Yoshida T, Yoshikawa Y: **Immunolocalization of proliferating cell nuclear antigen (PCNA) in cynomolgus monkey (Macaca fascicularis) testes during postnatal development.** *J Med Primatol* 2001, **30:**107-111.
59. Mayerhofer A, Bartke A: **Developing testicular microvasculature in the golden hamster, Mesocricetus auratus: a model for angiogenesis under physiological conditions.** *Acta Anat (Basel)* 1990, **139:**78-85.
60. Franck L I, Lissbrant E, Persson A, Damber JE, Bergh A: **Endothelial cell proliferation in male reproductive organs of adult rat is high and regulated by testicular factors.** *Biol Reprod* 2003, **68:**1107-1111.
61. Haggstrom RS, Wikstrom P, Jonsson A, Collin O, Bergh A: **Hormonal Regulation and Functional Role of Vascular Endothelial Growth Factor A in the Rat Testis.** *Biol Reprod* 2003, **69:**1231-1237.
62. O'Shaughnessy PJ, Baker P, Sohnius U, Haavisto AM, Charlton HM, Huhtaniemi I: **Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function.** *Endocrinology* 1998, **139:**1141-1146.
63. Baker P, Johnston H, Abel M, Charlton H, O'Shaughnessy P: **Differentiation of adult-type Leydig cells occurs in gonadotrophin-deficient mice.** *Reprod Biol Endocrinol* 2003, **1:**4-.
64. O'Shaughnessy PJ, Johnston H, Willerton L, Baker PJ: **Failure of normal adult Leydig cell development in androgen-receptor-deficient mice.** *J Cell Sci* 2002, **115:**3491-3496.
65. Teerds KJ, de Rooij DG, de Jong FH, van Haaster LH: **Development of the adult-type Leydig cell population in the rat is affected by neonatal thyroid hormone levels.** *Biol Reprod* 1998, **59:**344-350.
66. Mendis-Handagama SM, Ariyaratne HB, Teunissen van Manen KR, Haupt RL: **Differentiation of adult Leydig cells in the neonatal rat testis is arrested by hypothyroidism.** *Biol Reprod* 1998, **59:**351-357.