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Evidence for a GABAergic System in Rodent and Human Testis: Local GABA Production and GABA Receptors

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Key Words
Gamma-aminobutyric acid · Gamma-aminobutyric acid receptors · Leydig cell · Glutamate decarboxylase · Vesicular GABA transporter · Testis

Abstract
The major neurotransmitter of the central nervous system, gamma-aminobutyric acid (GABA), exerts its actions through $GABA_A$, $GABA_B$ and $GABA_C$ receptors. GABA and GABA receptors are, however, also present in several non-neural tissues, including the endocrine organs pituitary, pancreas and testis. In the case of the rat testis, GABA appears to be linked to the regulation of steroid synthesis by Leydig cells via $GABA_A$ receptors, but neither testicular sources of GABA, nor the precise nature of testicular GABA receptors are fully known. We examined these points in rat, mouse, hamster and human testicular samples. RT-PCR followed by sequencing showed that the GABA-synthesizing enzymes glutamate decarboxylase (GAD) 65 and/or GAD67, as well as the vesicular GABA transporter vesicular inhibitory amino acid transporter (VIAAT/VGAT) are expressed. Testicular GAD in the rat was shown to be functionally active by using a GAD assay, and Western blot analysis confirmed the presence of GAD65 and GAD67. Interstitial cells, most of which are Leydig cells according to their location and morphological characteristics, showed positive immunoreaction for GAD and VIAAT/VGAT proteins. In addition, several $GABA_A$ receptor subunits (α1–3, β1–3, γ1–3), as well as $GABA_B$ receptor subunits R1 and R2, were detected by RT-PCR. Western blot analysis confirmed the results for $GABA_A$ receptor subunits β2/3 in the rat, and immunohistochemistry identified interstitial Leydig cells to possess immunoreactive $GABA_A$ receptor subunits β2/3 and α1. The presence of $GABA_A$ receptor subunit α1 mRNA in interstitial cells of the rat testis was further shown after laser microdissection followed by RT-PCR analysis. In summary, these results describe molecular details of the components of an intratesticular GABAergic system expressed in the endocrine compartment of rodent and human testes. While the physiological significance of this peripheral neuroendocrine system conserved throughout species remains to be elucidated, its mere presence in humans suggests the possibility that clinically used drugs might be able to interfere with testicular function.

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Introduction

Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the vertebrate central nervous system. GABA interacts with ionotropic $GABA_A$ receptors, metabotropic $GABA_B$ receptors and a recently identified class of transmitter-gated ion channels, called $GABA_C$ receptors [1].

In addition to the well-established synaptic function of GABA, recent data indicate that GABA is important for other processes as well. For example, GABA is a factor involved in the regulation of neuronal cell proliferation during development [2–6]. Another non-synaptic role of GABA in neurons [7, 8], modulation of neurosteroid production, has also been described.

GABA is however, present also in non-neuronal tissues, including endocrine organs [9]. For example, the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) was identified in rodent and human pancreatic beta cells [10–13]. Recently, GABA was described to be produced by growth hormone cells of the anterior pituitary lobe [14]. Since $GABA_{A/B}$ receptor subunits were reported to be expressed in these and other endocrine tissues [15, 16], GABA may act as an auto-/paracrine factor in these organs [14, 17].

GABA is also present in the female and male reproductive tracts. Thus, GAD was detected by immunohistochemistry and other techniques in ovary and oviduct [18–21]. While a role for GABA in the female gonad is not well understood, a possible role of GABA in the oviduct is to activate ejaculated spermatozoa, which possess GABA receptors. In support of such a role, GABA leads to both initiation of the acrosome reaction and increased sperm motility [22–25]. These effects may be mediated via GABA receptors since $GABA_A$ receptors were identified on human spermatozoa [26, 27] and $GABA_B$ receptors have been found to be expressed by rat spermatozoa [16, 28].

A puzzling finding in this respect is that in addition to GABA receptors, GABA appears to be present in the male gonad. Since activation of testicular spermatozoa as a physiological function can be ruled out, GABA must have largely unexplored other intratesticular functions. The evidence for testicular GABA stems from Northern blot detection of GAD mRNA in rat [29] and mouse (nuclease protection assay) [12] active GAD in hamster (enzymatic assay) [29, 30] and mRNA/protein human testes [31]. Of the two major forms of GAD (GAD65 and GAD67), GAD67 and its splice variant GAD25, which, however, is not enzymatically active, are expressed in human testis [32]. Which testicular cells are able to synthesize GABA is unfortunately not fully known. Studies locating GAD mRNA suggested that the cellular sources of GABA may reside within the tubular compartment in germ cells [20, 31], but functional GAD protein has to our knowledge not been found. As mentioned above, GABA receptors are present on ejaculated spermatozoa. Since GABA receptor subunits have been identified by RT-PCR studies in rodent testis [15, 16, 28, 33], it is at present unclear whether they correspond to the ones observed in spermatozoa only, or whether other testicular cells may bear GABA receptors as well. The latter possibility is suggested by results in the rat, in which the production of androgens by Leydig cells was increased by GABA [34, 35], implying that these endocrine cells contain functional GABA receptors.

In the present study, we attempted to clarify this issue and have provided molecular details of sites of GABA production, storage and receptor-bearing targets in rodent and human testes.

Materials and Methods

Human Biopsies

Archival testicular biopsies from adult men (age range 22–44 years) were analysed. The biopsies (n = 3) used for this study revealed normal spermatogenesis with no or slight alteration. All biopsies had been fixed in Bouin's fixative and were embedded in paraffin. Sections (5 μm) were cut and used for immunohistochemical staining as described below. The evaluation of human specimens was approved by the Ethics Committee of the Technical University of Munich, Germany.

Animals

Testes were obtained from adult (Sprague-Dawley, Wistar) male rats (in total 29) and from adult (BALB/c) mice (in total 7; bred at the Technical University of Munich, Germany). According to the animal care guidelines, they were painlessly killed under ether anaesthesia by exsanguination and testes were rapidly removed. Testes were also obtained from adult male golden hamsters (n = 12) that had been raised at the Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina. Hamsters were housed in rooms at 23 ± 2°C and kept from birth in a long photoperiod (14:10 h light/dark, lights on from 7.00 to 21.00 h). Pelleted food and tap water were provided ad libitum. Hamsters were killed by decapitation and the testes were rapidly removed. The maintenance and treatment of the hamsters were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by a local Committee.

RNA Preparation and RT-PCR

Isolation of RNA from rodent testes, as well as RT and PCR for GAD65/67, VIAAT/VGAT (vesicular inhibitory amino acid transporter also known as vesicular GABA transporter, VGAT) and GABA receptor subunits were performed as described elsewhere [36]. Commercial human testicular cDNAs (pooled from 19 men; Clontech Inc., Palo Alto, Calif., USA; one sample from Invitrogen, Karlsruhe, Germany) were also used for PCR. Conditions of PCR amplification consisted of 30, 35 or 40 cycles (94°C for 30 s, 55°C

Table 1. Sequences of oligonucleotide primers used in RT-PCR studies

		Species	Size, bp	Primer
GAD65		R	422	5′-CAAGTGGAAGCTGAACGGTGT-3′ 5′-CTTCCAGAACTCGCAAACTAG-3′
		M	445	5′-CTTCTTCCGGATGGTCATCTC-3′ 5′-AGAGGTATTCTAAACTTAAGA-3′
GAD67		R, GH	440	5′-TGCAGTTCTTACTGGAGGTGG-3′ 5′-GATGCTGTACATGTTGGATAT-3′
		M	393	5′-CTTCTTCCGGATGGTCATCTC-3′ 5′-ACGAGCAACATGCTATGGTCT-3′
		Hum	624	5′-ATTCTTGAAGCCAAACAG-3′ 5′-TAGCTTTTCCCGTCGTTG-3′
VIAAT/VGAT		R, M	356	5′-CATTCAGGGCATGTTCGT-3′ 5′-CTATGATGGACCAGGACT-3′
		Hum	186	5′-GTATCTTGTACGTCGTGG-3′ 5′-GGATGTTGATGACGAAGTGGG-3′
$GABA_A$	α1	R, M	231	5′-CTACAGCAACCAGCTATACCC-3′ 5′-GCTCTCTGTTTAAATACGTGG-3′
		R (nested)	169	5′-AACTTAGGCCAGGGTGAC-3′ 5′-GATTCCAAATAGCAGCGG-3′
		Hum	357	5′-AGAGGTTATGCATGGGATGG-3′ 5′-GATCTATTGATGTGGTGTGG-3′
	α2	R, GH, M	282	5′-AAGGCTCCGTCATGATACAG-3′ 5′-ACTAACCCCTAATACAGGC-3′
	α3	R	418	5′-ACTTGCTTGGTCATGTTGTTGGG-3′ 5′-TTTCTTCATCTCCAGGGCCTCTG-3′
		M	418	5′-GACTTGCTTGGTCATGTTGTTGGG-3′ 5′-CAGAGGCCCTGGAGATGAAGAAGA-3′
		Hum	331	5′-GGTTCATAGCCGTCTGTTATGC-3′ 5′-TTGTAGGTCTTGGTCTCAGTCG-3′
	β1	M	540	5′-ATGATGCATCTGCAGCCA-3′ 5′-TGGAGTTCACGTCAGTCA-3′
		Hum	344	5′-AGCAAACAAGACCAGAGTGC-3′ 5′-AACATTCGGGACCACTTGTC-3′
	β2	Hum	424	5′-CATTGACATGTACCTGATGG-3′ 5′-ATCAGTCAAGTCAGGGATGG-3′
	β3	R, GH, M	224	5′-AGCCAAGGCCAAGAATGATCG-3′ 5′-TGCTTCTGTCTCCCATGTACC-3′
	γ1	R	191	5′-TTTCTTACGTGACAGCAATGG-3′ 5′-CATGGGAATCAGAGTAGATCC-3′
		M	191	5′-TTTCTTACGTGACAGCAATGG-3′ 5′-CATGGGAATGAGAGTGGATCC-3′
	γ2	R	351	5′-GCAATGGATCTCTTCGTC-3′ 5′-GTCCATTTTGGCAATGCG-3′
		M	351	5′-GCAATGGATCTCTTTGTA-3′ 5′-GTCCATTTTGGCAATGCG-3′
		Hum	329	5′-CAGCGATGGATCTCTTTG-3′ 5′-GTCCATTTTGGCAATGCG-3′
	γ3	R, M	251	5′-TGTCGAAAGCCAACCATCAGG-3′ 5′-GACTTGCACTCCTCATAGCAG-3′
$GABA_B$	R1	R, M, Hum	519	5′-GTACGTCTGGTTCCTCAT-3′ 5′-AGATCATCCTTGGTGCTG-3′
	R2	R, M	354	5′-CATCATCTTCTGCAGCAC-3′ 5′-TCTGTGAAGTTGCCCAAG-3′
		Hum	596	5′-ACCATCTCAGGAAAGACT-3′ 5′-CCTTATCATCCTTGGAGG-3′

R = Rat; M = mouse; GH = golden hamster; Hum = human.

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.

Immunohistochemistry

Testicular distribution of GAD65/67, VGAT, $GABA_A$-α, $GABA_A$-α1 and $GABA_A$-β2/3 was examined in rat and human testes using an avidin-biotin-peroxidase immunohistochemical method as described previously [37, 38]. Rat testes and other tissues (from adult Sprague-Dawley and Wistar rats) were fixed in Bouin's solution and embedded in paraffin. Archival testicular biopsies from men had been fixed in Bouin's fixative and embedded in paraffin. The following specific antibodies/antisera were employed: rabbit polyclonal antiserum to GAD65/67, which recognizes epitopes common to either form (DPC Biermann, Bad Nauheim, Germany; dilution 1:500); rabbit antiserum anti-VGAT (SySy Synaptic Systems GmbH, Göttingen, Germany; dilution 1:750); rabbit polyclonal antiserum anti-$GABA_A$-α1 (Alomone Labs Inc., Jerusalem, Israel; dilution 1:750); mouse monoclonal antibody anti-$GABA_A$-β2/3 (Upstate Biotechnology Inc., Lake Placid, N.Y., USA; dilution 1:500) [39]; mouse monoclonal antibody anti-$GABA_A$-α (Roche Diagnostics Inc., Mannheim, Germany; dilution 1:1,000); sheep monoclonal antibody anti-$GABA_B$-R1 (gift from Graham Disney and Fiona Marshall, Glaxo-Wellcome R&D Inc., Stevenage, UK; dilution 1:1,000–1:500), and sheep monoclonal antibody anti-$GABA_B$-R2 (GlaxoWellcome R&D; dilution 1:1,000–1:500). Sections incubated with buffer alone or buffer containing mouse or rabbit normal (i.e. non-immune) serum, respectively, served as controls for all samples. The sections were examined with a Zeiss Axiovert photomicroscope (Zeiss, Oberkochen, Germany).

Western Blotting

Tissues from rat testis (Sprague-Dawley) were homogenized in 62.5 m*M* 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). Then, 15 μg of protein per lane was loaded onto Tricine-SDS-polyacrylamide gels (12.5%), electrophoretically separated and blotted onto nitrocellulose as described previously [40]. Samples were probed with the same GAD65/67 and $GABA_A$-β2/3 antisera used for immunohistochemistry (incubation overnight at 4 °C, dilution 1:500). Immunoreactivity was detected using peroxidase-labeled antisera (Dianova, Hamburg, Germany; dilution 1:3,000) and enhanced chemiluminescence (Amersham Buchler, Braunschweig, Germany), as described elsewhere [41].

GAD Assay

GAD assays were performed as described previously [42] by using ^{14}C1-glutamic acid (Biotrend, Köln, Germany; specific activity 50–60 mCi/mmol). Rat testes and, as a positive control, cerebellum, were homogenized for use in GAD assays. The activity was expressed per microgram of protein. Protein samples of rat testes heated to 95 °C for 5 min served as negative controls. Results obtained from testes are given as a percentage of activity determined in the cerebellum. Results obtained were analysed statistically with a computer program (Prism, GraphPad Software Inc., San Diego, Calif., USA). We performed one-way analysis of variance by ANOVA followed by Student-Newman-Keuls test for multiple comparisons and Student's t test. Data were expressed as mean ± standard error of the mean (SEM) and $p < 0.05$ was considered significant.

Laser Microdissection and RT-PCR

Rat testes embedded in paraffin were cut into sections (5 μm) and mounted onto a 1.35-μm thin polyethylene naphthalene membrane pasted to a glass slide which had been pretreated with UV light for 30 min. The sections were deparaffinized and processed for haematoxylin staining. Laser microdissection (LMD) was performed as previously described [17, 43, 44]. In brief, employing a nitrogen laser of the Robot-MicroBeam (P.A.L.M. GmbH Mikrolaser Technologie, Bernried, Germany), groups of interstitial cells were circumscribed and thus isolated from the surrounding tissue. This microdissected sample was ejected from the object slide and catapulted directly into the cap of a microfuge tube. Fifty microlitres of RNA stabilization reagent (RNEasy Protect Mini-Kit, Qiagen, Hilden, Germany) were added into the cap. Samples were frozen at –70 °C until RNA extraction (RNEasy, Qiagen). RT followed by two nested PCR amplifications were performed. To test for specificity, in the consecutive section, interstitial cells in a given area were eliminated by a few directed laser shots, the area was circumscribed and the remaining material was catapulted into the cap of a microfuge tube and used as negative control in the RT-PCR assays.

Results

The Genes for GAD65/67 and VIAAT/VGAT Are Expressed in the Testes of Rodents and Humans

RT-PCR followed by sequencing showed that genes of GAD are expressed in the testes of rat, mouse, hamster and human (fig. 1A). We detected GAD65 and GAD67 in the testes of rat and mouse, while in human testes, only GAD67 was found. The study in hamsters may have been hampered due to the fact that oligonucleotide primers for PCR corresponding to rat and murine primers, respectively, were used because DNA sequences of the hamster GAD isoforms were not known. However, partial sequences obtained from analysis of hamster testes and brain indicated sequence homology to rat GAD67 (position 690–721, GenBank accession number M76177). Using oligonucleotide primers targeted specifically for VIAAT/VGAT (murine/human sequence), we obtained positive results in rat, murine and human testes (fig. 1B). With these oligonucleotide primers, VIAAT/VGAT was not found in hamster. Western blots performed with rat testicular homogenates and antiserum to GAD65/67 (fig. 1C) confirmed the presence of both GAD65 and GAD67 in rat testis.

GAD Is Active in Rat Testis

Evidence for enzymatically active GAD in rat testis was provided by measurements of ^{14}C1-glutamic acid decarboxylation. Tissue of the cerebellum was used as a positive control (100%) and samples of cerebellum and testes heated to 95 °C for 5 min served as negative con-

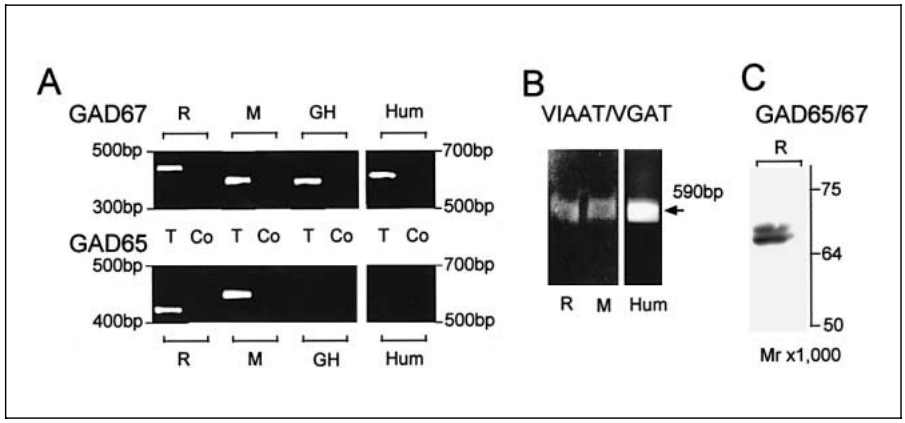

Fig. 1. Expression of GAD isoforms and VIAAT/VGAT in testes of rat (R), mouse (M), golden hamster (GH) and human (Hum). **A** Ethidium bromide-stained agarose gels depict results of RT-PCR for GAD. GAD67 is expressed in the testis of all species investigated. In addition, rat and murine testes also possess GAD65. PCR reactions without template served as controls (Co). **B** RT-PCR for VIAAT/VGAT in rat (R), mouse (M) and human (Hum) testis. Sequencing of RT-PCR products confirmed their identity. **C** Western blot of rat (R) testis probed with antiserum against GAD65/67 revealed the presence of both proteins.

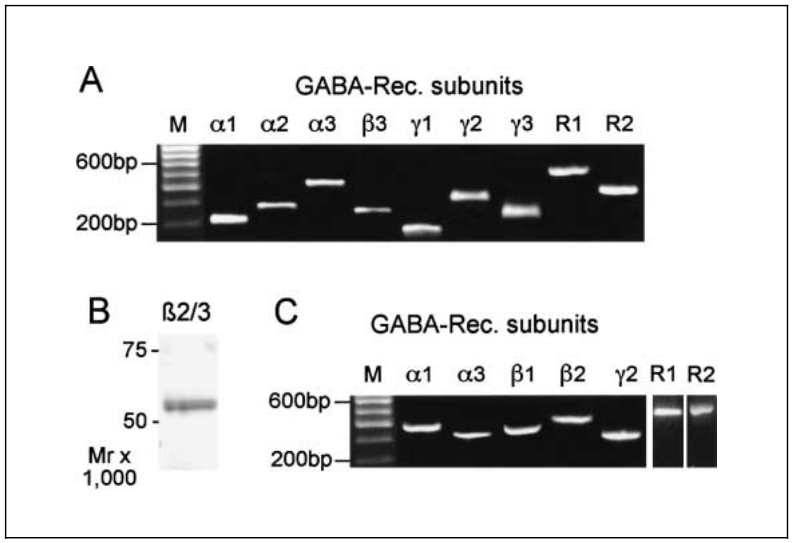

Fig. 2. $GABA_A$ and $GABA_B$ receptor subunits in rat and human testes. Ethidium bromide-stained agarose gels show results of RT-PCR analyses of rat (**A**) and human (**C**) testis. **A** In rat testis, $GABA_A$ receptor subunits α1 (231 bp), α2 (282 bp), α3 (418 bp), β3 (224 bp), γ1 (191 bp), γ2 (351 bp) and γ3 (251 bp) and $GABA_B$ receptor subunits R1 (519 bp) and R2 (354 bp) were detected. **C** The $GABA_A$ receptor subunits α1 (357 bp), α3 (331 bp), β1 (344 bp), β2 (424 bp) and γ2 (329 bp), as well as $GABA_B$ receptor subunits R1 (519 bp) and R2 (596 bp) are present in human testis. **B** Western blots of rat testis probed with an antiserum recognizing both $GABA_A$-β2 and 3 confirmed their presence (β2: 55,000 molecular mass; β3: 57,000 molecular mass).

Table 2. Summary of RT-PCR results: distribution of GABA receptor subunits in rat, mouse, hamster and human testis

	Rat	Mouse	Hamster	Human
GABA receptor subunit				
α1	+	+		+
α2	+	+	+	
α3	+	+		+
β1		+		+
β2				+
β3	+	+	+	
γ1	+	+		
γ2	+			+
γ3	+	+		
R1	+	+		+
R2	+	+		+

+ indicates PCR product of the corresponding subunit, which was confirmed by sequencing.

trols. Testes of adult rats (n = 11) showed 3.35 ± 0.161% (mean ± SEM) of the GAD activity obtained in cerebellum. Heating of the samples completely reduced this value to background levels.

Identification of Testicular GABA Receptor Subunits by RT-PCR and Western Blotting

Table 2 shows details of results obtained in rat, mouse, hamster and human testes. In the case of rat testis, $GABA_A$ receptor subunits α1–3, β3 and γ1–3 and $GABA_B$ receptor subunits R1/2 are present (fig. 2A). Novel sequence information about $GABA_A$-α2 obtained from analysis of hamster testes (representing 2 independently derived identical sequences) was submitted to GenBank (accession number AF533532). This partial sequence shows 97% homology with rat, 95% homology with mouse and 84% homology with human $GABA_A$-α2 at the nucleotide level. Sequencing of 3 hamster $GABA_A$-β3 RT-

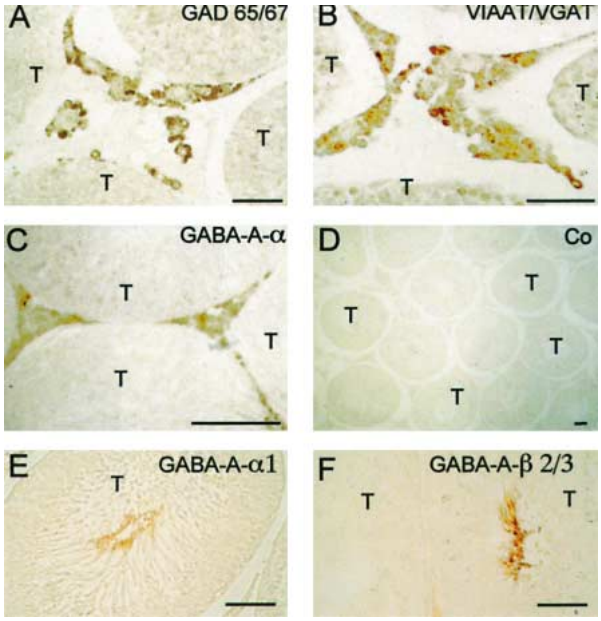

Fig. 3. Localization of GAD65/67, VIAAT/VGAT and $GABA_A$ subunit α in adult rat testis. Interstitial cells, presumably Leydig cells, located between the seminiferous tubules (T) possess immunoreactivity for GAD (**A**) and VIAAT/VGAT (**B**). Testicular sections of adult rat probed with an antibody against $GABA_A$-α also show interstitial staining (**C**). Elongated spermatids were also immunoreactive for $GABA_A$-α1 (**E**) and $GABA_A$-β2/3 (**F**). No reaction is observed in testis sections incubated with buffer or non-immune serum (not shown) instead of the primary antibody (**D**). Bars = 50 μm.

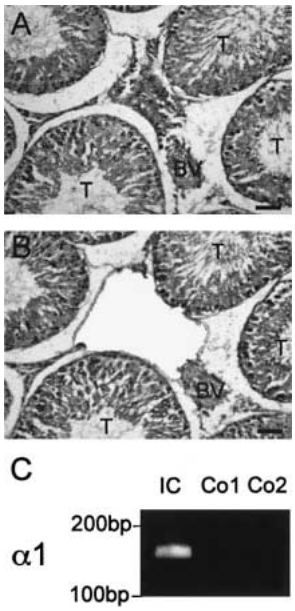

Fig. 4. Example of an experiment using LMD of grouped interstitial cells from a testicular section of an adult rat. **A**, **B** Consecutive testes sections prior to and after LMD. T = Seminiferous tubule; BV = blood vessel. Bars = 50 μm. In **B**, interstitial cells, presumably Leydig cells, were dissected and used for RT-PCR. **C** Interstitial cells (IC) possess mRNA for $GABA_A$ receptor subunit α1 (169 bp). To show specificity, these cells were selectively destroyed by laser shots prior to LMD, and the remaining tissue was dissected, recovered and used for RT-PCR (Co1). In addition, RT-PCR was performed without adding a template (Co2).

PCR products revealed complete identity with the corresponding rat sequence. Figure 2B depicts a Western blot of rat testis probed with $GABA_A$-β2/3 antibody, showing immunoreactive signals for both subunits. In human testis, RT-PCR results revealed that the $GABA_A$ receptor subunits α1/3, β1/2 and γ2, as well as $GABA_B$ R1 and R2 (fig. 2C, table 2), are expressed.

Cellular Localization of GAD65/67, VIAAT/VGAT and $GABA_A$ Receptor Subunits in Adult Rat Testes

To identify testicular sources of GAD, VIAAT/VGAT and GABA receptors ($GABA_A$-α, $GABA_A$-α1 and $GABA_A$-β2/3), immunohistochemistry was performed. The GAD antiserum employed recognizes both GAD65 and GAD67. We found that cells in interstitial spaces of rat testis were immunoreactive for GAD65/67 and VIAAT/VGAT. Rat testis showed interstitial immunostaining with anti-$GABA_A$-α, which does not distinguish between $GABA_A$-α subtypes. The staining pattern obtained for GAD65/67, VIAAT/VGAT and $GABA_A$-α was robust and observed in almost all interstitial spaces in rat testes (fig. 3A–C). Staining obtained with the antibody against $GABA_A$-β2/3 and anti-$GABA_A$-α1 (data not shown) was less robust, but specific, and was also seen in cells with the typical Leydig cell morphology and location. All controls performed were negative (fig. 3E).

In addition, spermatids immunoreactive for $GABA_A$-α1 and $GABA_A$-β2/3 (data not shown, fig. 3D, E, F) were seen inside the seminiferous tubules of rat testis.

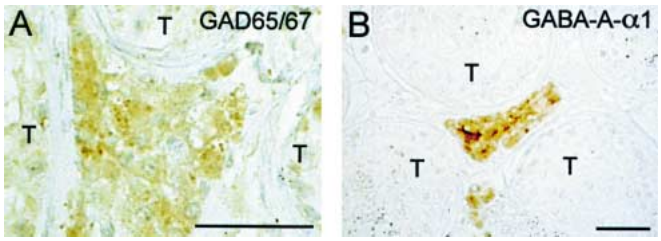

Fig. 5. In human testes, interstitial cells located between seminiferous tubules (T) are immunopositive for GAD (**A**) and $GABA_A$-α1 (**B**). Bars = 50 μm.

Rat interstitial cells, dissected by LMD from paraffin sections of adult rat testes, were isolated and analysed by RT-PCR. Figures 4A and B show consecutive testicular sections, one of them after LMD (fig. 4B). Dissected samples, in which interstitial cells were selectively destroyed by laser shots, served as negative controls. We designed oligonucleotide primers for nested RT-PCR analysis for $GABA_A$-α1. The results revealed that interstitial cells of adult rats express $GABA_A$-α1 (fig. 4C) and are in line with our immunohistochemical results, also indicating the presence of a $GABA_A$-α subunit.

Localization of GAD65/67 and $GABA_A$ Receptor Subunit Proteins in Human Interstitial Cells

Immunohistochemistry was performed on sections of human biopsies to investigate the cellular localization of GAD, VIAAT/VGAT and GABA receptors. Consistent with the results in rat, we found interstitial cells immunoreactive for GAD and $GABA_A$-α1 (fig. 5A, B). Furthermore, weak immunoreactivity for $GABA_A$-β2/3 was seen in the interstitium. Labelling was absent in all controls. Successful immunohistochemical analysis of human samples with available antibodies against VIAAT/VGAT, $GABA_B$-R1 and $GABA_B$-R1 was unfortunately not possible, like due to the suboptimal fixation/embedding conditions of these samples.

Discussion

The current study, by identifying at the molecular level the components of testicular GABA synthesis, storage and its receptors, shows that a testicular GABAergic system exists in the interstitial, i.e. endocrine, compartment of the testes of rodents and humans.

Our results reveal that the crucial enzymes for GABA synthesis are present in the testes of several species. Marked species differences became apparent with respect to the GAD forms. Thus, GAD65 and/or GAD67 were found. We made no attempt to examine whether splice variants are expressed, mainly because the previously described testicular variant GAD25 [32] appears not to be enzymatically active. Instead, testicular GAD65/67 forms in the rat were enzymatically active in our study, a result in line with previous studies in hamster testis using a comparable GAD assay technique [30]. The study in hamster did not localize GAD to testicular cells, but other investigators examining rat and human testes [20, 31] found GAD mRNA in spermatids and germ cells. The techniques employed were in situ hybridization and RT-PCR techniques, while we are not aware of techniques which localize the corresponding GAD protein with one exception. Our current study thus contrasts to these reports, since we did not find immunoreactive GAD with an antiserum recognizing both GAD65 and GAD67 in the germinal epithelium of the human, rat, mouse and hamster testes, but rather in the interstitial compartment. The reasons for these differences are currently not known, but we speculate that either the abundance of GAD protein in the tubular compartment is very low or that a splice variant, including GAD25, may be present inside the seminiferous tubules, which is not recognized by the antiserum used. The staining pattern resulting from the use of the antiserum to GAD65/67 was also found when, in addition, antiserum recognizing VIAAT/VGAT was used. This further substantiates our conclusion that testicular interstitial cells of all species examined may be able to produce and store GABA.

The action of GABA requires GABA receptors. Indeed, several $GABA_A$ and $GABA_B$ receptor subunits were found in testes in the present and in previous studies. Our results are largely in accord with a study performed in rat testes. Akinci and Schofield [15] also found $GABA_A$ subunits α1–3, β3 and γ1/2. In contrast to their work, we did not find $GABA_A$ subunits β1/2 in rat testis. Additionally, we identified $GABA_A$ subunit γ3, a subunit not reported by Akinci and Schofield [15] to be present in rat testis. Whether methodological distinctions or differences in cell activity, hormonal or developmental state [45–47] may account for these small discrepancies remains to be shown. Our results concerning subunits of the $GABA_B$ receptor in testis tally with RT-PCR studies made by He et al. [28] in rat testis and extend these results to other rodent species and human testis. Thus, testicular tissue of different species contains mRNA of two distinct classes of GABA receptors.

With both GABA-synthesizing/storing enzymes and GABA receptors present in the testis, what is the role of this non-neuronal GABAergic system in the male gonad?

A first possibility to be considered is regulation of endocrine function, since interstitial cells bear GABA receptors. Indeed, evidence for a role of GABA in the regulation of Leydig cell function has been provided. GABAergic drugs (including benzodiazepines) and GABA were reported to modulate basal and gonadotrophin-stimulated androgen production of Leydig cells in rat [34, 35, 48]. Our immunohistochemical results indicate that the majority of the testicular interstitial cells, which are both sources and targets of GABA, are typical Leydig cells. It is therefore possible that GABA may act as

an autocrine or paracrine regulator of endocrine function in the testis, a role as described for other endocrine organs, including pancreatic islets [49, 50] or pituitary [14, 17]. While the function of GABA produced in the pituitary remains to be fully elucidated, GABA secreted by pancreatic B cells serves as a paracrine inhibitory factor for glucagon production [51]. The targeted A cells of the pancreatic islets express $GABA_A$ receptors.

A second possibility, which cannot be ruled out, is that testicular GABA may participate in the maturation and differentiation of germ cells. It is unknown whether GABA produced by interstitial cells, as indicated by our results, can reach the tubular compartment. However, it was suggested that GABA could also be produced in this compartment. Evidence for this assumption was the detection of GAD mRNA in spermatids and germ cells [20, 31]. However, whether enzymatically active GAD is indeed translated from this mRNA is not known. Importantly, we did not find GAD protein in the tubular compartment in the present study. Rat and human germ cells bear GABA receptors [22–27], and our immunohistochemical studies reveal immunoreactivity for $GABA_A$-α1 and $GABA_A$-β2/3 on rat spermatids at least in some testicular sections. Whether these receptors are functional at this developmental stage awaits future proof. Therefore, information available at the present time does not allow us to decide whether GABA might influence maturation of germ cells and spermatids in the testis. Clear evidence, however, was provided for functional GABA receptors in ejaculated spermatozoa (acrosome reaction [23–25] and regulation of human sperm motility [22]). The source of GABA in this case resides within the oviduct. Thus, we speculate that GABA receptors are expressed already during germ cell development in preparation for later activation by GABA present in the oviduct [9, 18–21, 52–57].

A third possibility to be considered is that GABA may serve as a trophic factor initiating and controlling cell proliferation and/or differentiation in the interstitial compartment. Such an influence was shown for developing neurons in the central nervous system [2, 3, 58]. In human and mouse testes, Leydig cells proliferate, albeit at a low level, throughout adulthood [59, 60]. It remains to be shown whether GABA contributes to proliferation and/or differentiation and function of Leydig cells.

While the physiological consequence of the presence of GABA in the testis is currently not known, our data may bear two potential clinical implications for humans. First, the presence of a GABAergic system in the testis indicates as yet unrecognized possible targets for drugs interacting with GABA metabolism and/or GABA receptors, which are widely used in humans. Unexplored side effects therefore are a possibility warranting further investigation.

Second, autoantibodies against GAD have been found to be associated with several human diseases, including insulin-dependent diabetes mellitus, neurological diseases [61] and autoimmune syndromes [10, 62]. Patients with autoimmune polyglandular syndrome type II, for example, are reported to have autoantibodies against Leydig cells and may present hypogonadism [63] or subclinical symptoms [64]. Whether, aside from these diseases, autoantibodies to GAD may be associated with or even be the cause of certain alterations of testicular function in humans is a possibility which to our knowledge has not yet been explored.

In summary, testicular interstitial cells produce GABA and express GABA receptors. Since the components of this novel GABAergic system exist in the endocrine compartment of the rodent and human testis, our work presented in this study prepares the ground for future studies to analyse the physiological role and clinical implications of this peripheral 'neuroendocrine' system.

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