Epithelial Localization of Green Fluorescent Protein-Positive Cells in Epididymis of the GAD67-GFP Knock-in Mouse

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ABSTRACT: γ-Aminobutyric acid (GABA), which is a major inhibitory neurotransmitter in the brain, is also found in many peripheral nonneuronal tissues, including male reproductive organs. However, the distribution of GABAergic cells in various organs is not known. The GAD67-GFP knock-in mouse is a useful model for studying the distribution and morphology of GABAergic neurons in the brain. We examined the male reproductive organs of GAD67-GFP knock-in mice by fluorescence microscopy and found cells with strong green fluorescent protein (GFP) signal exclusively in the epithelium of the initial segment and proximal caput of the epididymis. The characteristic cell morphology suggested that these were narrow cells. These GFP-positive narrow cells also expressed GAD67 and GABA. Reverse transcription polymerase chain reaction (RT-PCR) analysis showed that the predominant glutamic acid decarboxylase (GAD) isoform expressed in the epididymis is GAD67. RT-PCR analysis also revealed that mRNAs encoding the $GABA_A$ and $GABA_B$ receptor subunits necessary for the assembly of functional receptors are expressed in the epididymis. $GABA_A$ receptor subunit mRNAs detected in the proximal epididymis included $\alpha2$, $\beta1$, $\gamma1$, and $\gamma3$, and both the R1 and R2 subunit mRNAs of $GABA_B$ receptors were detected. Immunohistochemical analysis of $GABA_A$ receptor subunit proteins revealed that $\alpha2$, $\beta1$, and γ subunits expressed in spermatozoa, whereas we did not detect these $GABA_A$ receptor subunits in epithelial cells. $GABA_B$ receptors were produced by narrow cells and spermatozoa of GAD67-GFP knock-in and wild-type Jcl:ICR mice. Our data suggest that the GABA system might have important functional roles in narrow cells and on spermatozoa in the lumen.

Key words: Narrow cell, GABA, immunohistochemistry, $GABA_A$ receptor, $GABA_B$ receptor.

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γ-Aminobutyric acid (GABA) is a principal inhibitory neurotransmitter in the adult mammalian brain. GABA is also detected in many peripheral nonneuronal tissues (Watanabe et al, 2002). High levels of GABA have been observed in the male reproductive system, particularly in the testis, epididymis, vas deferens, seminal vesicle, and prostate gland (Erdö et al, 1983; Erdö and Kiss, 1986; Leader et al, 1992; Frungieri et al, 1996). It has been suggested that GABA participates in endocrine function in the testis. GABA stimulates production of testosterone in the testis (Ritta and Calandra, 1986; Ritta et al, 1991) and might also be important in the regulation of sperm activities, including agglutination and motility (Barna and Boldizsár, 1996; Calogero et al, 1996). GABA

exerts its effects through 2 types of receptors: ionotropic $GABA_A$ and $GABA_C$ receptors and metabotropic G-protein–coupled $GABA_B$ receptors.

$GABA_A$ receptors have been detected on sperm, suggesting that these receptors mediate the progesterone-initiated acrosome reaction (Hu et al, 2002a,b). This receptor is also found in seminal vesicles and the lateral lobe of the prostate gland (Napoleon et al, 1990; Collier et al, 1992). $GABA_B$ receptor mRNAs were detected in rat testis and sperm by reverse transcription-polymerase chain reaction (RT-PCR), and it is thought that these receptors contribute to the ability of spermatozoa to fertilize eggs and are involved in induction of the acrosome reaction (He et al, 2001, 2003). Despite significant amounts of data for GABA receptors in the male reproductive system, there is no information regarding localization of GABAergic cells in the male accessory reproductive organs.

GABA is synthesized primarily from glutamic acid by a decarboxylation reaction catalyzed by glutamic acid decarboxylase (GAD). Mammals express 2 isoforms of GAD, GAD65, and GAD67, which are encoded by 2 distinct genes (Watanabe et al, 2002). In the brain, GAD67 is responsible for synthesis of more than 90% of GABA

and is a soluble cytosolic protein, whereas GAD65 is preferentially localized near neuronal synaptic vesicles (Soghomonian and Martin, 1998). To study the precise distribution, morphology, electrophysiologic properties, and development of GABAergic neurons, a GAD67–green fluorescent protein (GFP) knock-in mouse (GAD67-GFP knock-in mouse) was developed (Tamamaki et al, 2003; Tanaka et al, 2003; Jiang et al, 2004). We examined the male reproductive organs of this transgenic mouse by fluorescence microscopy and observed cells with strong GFP signals in the initial segment and proximal caput of the epididymis. Here, we characterize expression of GAD67, GABA, and GABA receptors in GFP-positive cells in the epididymis of GAD67-GFP knock-in mice.

Materials and Methods

Animals

The generation of the GAD67-GFP knock-in mice (Δneo) used in this study is described by Tamamaki et al (2003). In brief, a cDNA encoding enhanced GFP (EGFP; Clon Tech, Palo Alto, Calif) was introduced into exon 1 of the GAD67 gene by homologous recombination to express EGFP specifically in GAD67-positive cells of the transgenic mice. Recombinant embryonic stem cells were used to generate chimeric mice by 8–cell stage injection. GAD67-GFP knock-in mice were obtained by breeding chimeric male mice with C57BL/6 female mice. The GAD67-GFP knock-in mice, which retain a loxP-flanked neomycin-resistant cassette (PGK-Neo), were mated with CAG-cre transgenic mice (Sakai and Miyazaki, 1997) to delete the PGK-neo sequences. The resultant GAD67-GFP knock-in (Δneo) mice lack the PGK-Neo cassette, and the expression of EGFP in GAD67-GFP knock-in (Δneo) mouse brain is higher than that of EGFP in GAD67-GFP knock-in mouse brain (Tamamaki et al, 2003). In this study, we used the GAD67-GFP knock-in (Δneo) mice and refer to them simply as GAD67-GFP knock-in mice. EGFP was found to be expressed specifically in GAD67-positive neurons (ie, GABA neurons) of GAD67-GFP knock-in mice (Tamamaki et al, 2003). These mice are maintained at the Department of Anatomy, Osaka Medical College (Osaka, Japan). Male wild-type Jcl:ICR and C57BL/6 mice (8 weeks old) were obtained from Clea Japan (Osaka, Japan). All animals were housed in a temperature-controlled room (23°C) and allowed water and regular food (CE-2, Clea Japan) ad libitum. A standard dark/light schedule of 12/12 hours was used. All animal experiments were reviewed and approved by the Ethics Review Committee for Animal Experimentation of Osaka Medical College.

GFP Fluorescence Observation

Thirty adult male GAD67-GFP knock-in mice aged 4 weeks to 4 months and 3 mice aged 7 months were used for analysis of the distribution of GFP in reproductive organs, including the testis, epididymis, vas deferens, prostate gland, and preputial gland. Mice were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and perfused with Ringer solution via the left ventricle and then with 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The reproductive organs were removed and postfixed for 5 hours in the same fixative at 4°C. The specimens were then rinsed with PBS and immersed in 30% sucrose for cryoprotection. Specimens were embedded in OCT compound (Miles, Elkhart, Ind). The blocks were cut into 10 sections (30 μm thick) with a cryostat (Leica CM3050, Nussloch, Germany), air-dried at room temperature, and mounted with aqueous/dry mounting medium (Crystal/Mount, Biomeda, Foster City, Calif). Some sections were stained with propidium iodide (Molecular Probes, Eugene, Ore) after treatment with 0.01% RNase (Type IIIA, Sigma, St Louis, Mo) in PBS for 60 minutes at 37°C. To identify epididymal segments, such as the initial segment, proximal caput, distal caput, corpus, and cauda, and cells with GFP signal, the epididymis was stained with toluidine blue, hematoxylin-eosin, and the periodic acid-Schiff reaction.

Fluorescence was observed and photographed with a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan) equipped with a digital camera (Leica DC300F, Leica) or with a confocal laser microscope (Radiance 2000, Bio-Rad Laboratories, Hercules, Calif, or LSM510, Carl Zeiss Co, Ltd, Oberkochen, Germany).

RNA Isolation and RT-PCR

For RT-PCR analysis of GAD65, GAD67, and GABA$_A$ and GABA$_B$ receptor subunits, total RNA was isolated from whole epididymis and whole brain of 8-week-old male GAD67-GFP knock-in mice with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For GABA$_A$ and GABA$_B$ receptor subunits, epididymides sectioned into initial segment, caput, corpus, and cauda regions were also analyzed. Total RNA from 30 mg of tissue was finally eluted with 50 μL of RNase-free water. Subsequently, cDNA was synthesized with AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Sciences, St Petersburg, Fla) according to the manufacturer's instructions. Briefly, total RNA solution (17 μL) was incubated with Oligo-d(T)$_{12-18}$ primer (0.05 mg) at 70°C for 10 minutes. Reverse transcription was carried out at 41°C for 60 minutes in 25 μL of the supplied buffer containing 17 μL of the RNA-primer mixture, 0.25 mM dithiothreitol, 12.5 U of RNase inhibitor, and 25 U of AMV reverse transcriptase. An aliquot of the cDNA was subjected to PCR amplification. The reaction mixture (50 μL) consisted of 1 U Ex *Taq* polymerase (Takara Shuzo, Shiga, Japan), 0.2 μM dNTP mixture, 3 μL cDNA solution, and 0.2 μM of each primer. The sequences of primer pairs for mouse GAD65, GAD67, and GABA$_A$ and GABA$_B$ receptor subunits are shown in Table 1. Reactions were amplified in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif) with preincubation at 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 60°C for 50 seconds, and 72°C for 100 seconds. The amplification finished with a single 7-minute incubation at 72°C. The PCR products were separated on 1.5% agarose gels. Gels were stained with 0.1% ethidium bromide, visualized by ultraviolet transillumination, and documented with black and white instant film.

Immunohistochemistry of GAD67, GABA, and GABA Receptor Subunits

Eight-week-old male GAD67-GFP knock-in and Jcl:ICR mice (n = 3 each strain) were anesthetized with pentobarbital. For

Table 1. *Primer sequences used for reverse transcription polymerase chain reaction of mouse GAD65, GAD67, and GABA$_A$ and GABA$_B$ receptor subunits*

Primer	Sequences	Product Size, bp	GenBank Accession No.
GAD65	F 5′-AGCCTTAGGGATTGGAACAG-3′ R 5′-TTCCGGGACATCAGTAAC-3′	258	L16980
GAD67	F 5′-GATACTTGGTGTGGCGTAGCCC-3′ R 5′-ACGGGTGCAATTTCATATGTGAACATA-3′	575	Y12257
α1	F 5′-CAGCAAGAACTGTCTTTGGAG-3′ R 5′-GCATACCCTCTCTTGGTGAA-3′	190	NM_010250
α2	F 5′-TCAGTGCTCGAAATTCCCT-3′ R 5′-GTATCATGACGGAGCCTTTC-3′	195	NM_008066
α3	F 5′-ATGTCGTCATGACAACCCA-3′ R 5′-GTTTCTGGCACTGATACTCAAG-3′	197	NM_008067
α5	F 5′-GTCTCCCTCTCAACAACCT-3′ R 5′-GTAGAGAAGCGTGCCATCA-3′	140	AF540386
α6	F 5′-TGGGCAAACAGTTTCTAGTG-3′ R 5′-AGCACTGATGCTTAAGGTG-3′	238	NM_008068
β1	F 5′-TGGCTATACCACGGATGAC-3′ R 5′-AGTGTGGAAGGCATGTAGG-3′	222	NM_008069
β2	F 5′-GGCTACTTCATCCTGCAGA-3′ R 5′-GAAGGGCCATAAAGACGAAG-3′	235	NM_008070
β3	F 5′-GCCATCGACATGTACCTGA-3′ R 5′-GAATTCCTGGTGTCACCAAC-3′	260	NM_008071
γ1	F 5′-CGGATGGGCTATTTCACGA-3′ R 5′-CATTGCTGTCACGTAAGAAACC-3′	195	NM_010252
γ2	F 5′-ACTTCCCAATGGATGAACAC-3′ R 5′-TGTCATGGTCAGGACAGTAG-3′	375	M86572
γ3	F 5′-TCAAGCTGTCGAAAGCCA-3′ R 5′-GTCTTTGCCATCCAGACACT-3′	219	NM_008074
δ	F 5′-GCCAGAGTATCTCTAGGCA-3′ R 5′-CGTTCCTCACATCCATCTC-3′	244	NM_008072
R1a	F 5′-GAAGGTGGCATCAGG-3′ R 5′-CTTGGAGCAGATTCGG-3′	195	AF114168
R1b	F 5′-TGCCTCTTCTGCTGGTGATG-3′ R 5′-TCTCTGCGGCTGTTAACGTC-3′	210	AF120255
R2	F 5′-TCCATCATGGGCCTCATG-3′ R 5′-TCGCAGGTCCAGGAAGTA-3′	138	AF058795

GAD67, GABA, and GABA$_B$ receptor immunostaining, the anesthetized mice were perfused with 4% paraformaldehyde (and 0.1% glutaraldehyde for GABA) in PBS. Epididymides were dissected and immersed in the same fixatives for 4 hours at 4°C. Specimens were rinsed with PBS and then immersed in 30% sucrose. For GABA$_A$ receptor immunostaining, epididymides were dissected from the anesthetized mice without perfusion, frozen in liquid N$_2$, cryosectioned, throw-mounted on glass slides, air-dried, fixed with 4% paraformaldehyde in PBS for 5 minutes, and rinsed with PBS. After preparing the 10-μm-thick sections as described above, sections for GAD67 and GABA$_A$ receptor subunit immunostaining were incubated with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 60 minutes at 37°C in a humidified chamber to control for nonspecific reactions. Sections were then incubated at 4°C overnight with rabbit anti-GAD67 polyclonal antibody (diluted 1:1000; Chemicon International, Temecula, Calif) or goat anti-GABA$_A$ receptor α2, β1, γ1/2/3 polyclonal antibodies (diluted 1:100; Santa Cruz Biotechnology Inc, Santa Cruz, Calif). For GABA immunostaining, 10-μm-thick sections were preincubated with 1% sodium borohydride in PBS for 30 minutes at room temperature, washed in PBS, and incubated with Block Ace. The

sections were then incubated with rabbit anti-GABA polyclonal antibody (diluted 1:2000; Chemicon International) overnight at 4°C. After incubation with primary antibody, all sections were rinsed in PBS and incubated with Alexa 488–conjugated goat anti-rabbit IgG or Alexa 546–conjugated goat anti-rabbit IgG (diluted 1:300; Molecular Probes) for 60 minutes at room temperature. GABA$_B$ subunit immunostaining was performed as described previously (Kanbara et al, 2005). Some sections were stained with propidium iodide as described above. As negative controls for immunostaining, sections were incubated with nonimmune serum instead of primary antibody. All controls showed the expected negative results.

Results

Distribution of GFP-Positive Cells

In reproductive organs of 4-week- to 3-month-old GAD67-GFP knock-in mice, GFP-positive cells were found exclusively in the initial segment and proximal caput of the epididymis (Figure 1), and no GFP fluorescence
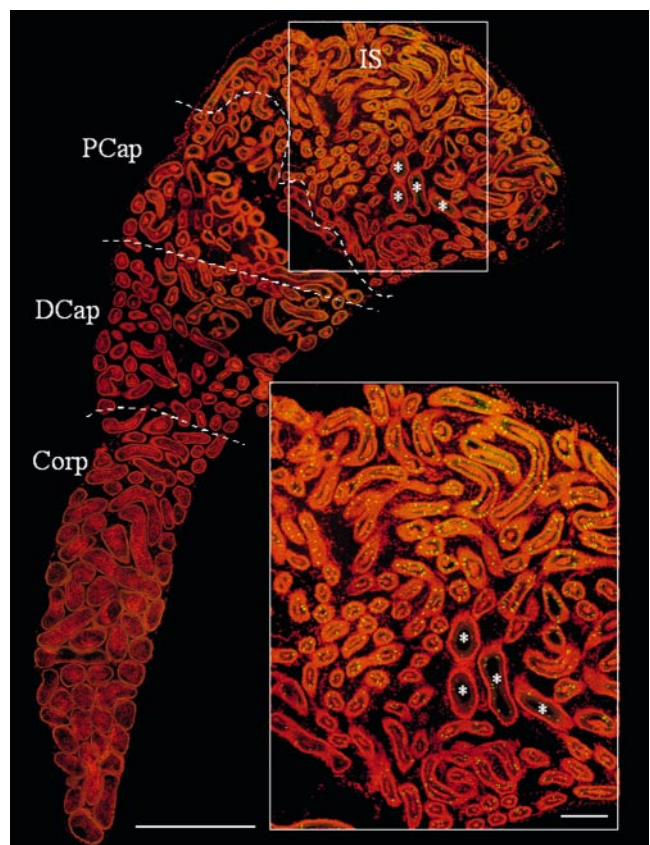
Figure 1. Low-power fluorescent micrograph montage illustrating the subdivisions of the proximal end of the epididymis and distribution of green fluorescent protein (GFP)–positive cells in GAD67-GFP knock-in mouse (8 weeks old). Nuclei are stained red with propidium iodide (PI). The initial segment (IS), proximal caput (PCap, * in inset), distal caput (DCap), and corpus (Corp) are indicated. Note strong GFP fluorescence (green) is observed exclusively in the initial segment and proximal caput of the epididymis. Scale bars = 1 mm; 250 μm in inset.

Figure 2. Enlarged micrographs of green fluorescent protein (GFP)–positive cells in the initial segment of the epididymis of GAD67-GFP knock-in mouse (8 weeks old). **(a)** GFP fluorescence (green); **(b)** Propidium iodide (PI) staining of nuclei (red); **(c)** differential interference contrast image; **(d)** merged image of **Panels a–c**. GFP-positive cells have the morphology characteristic of narrow cells. These cells are goblet-shaped with a basal foot (arrowhead in **Panel a**) and a thin infranuclear region. The nucleus is located more apically than in other cell types. The apical portion of the cells protrudes into the lumen (arrows in **a, d**). Scale bar = 50 μm.

was observed in the testis, vas deferens, seminal vesicle, prostate gland, or preputial gland. GFP fluorescence was not observed in the epithelium of distal caput, corpus, and cauda of the epididymis.

The GFP-positive cells were goblet shaped, with a basal foot and a thin infranuclear region (Figure 2). The nucleus was located more apically than in other cell types (Figure 2b and d). The apical portion of the cells protruded into the lumen (Figure 2d). The GFP-positive cells in the serial section were stained intensely with toluidine blue, as has been observed by Sun and Flickinger (1980). On the basis of this morphology, these cells were identified as narrow cells. The distribution and fluorescence intensity of these GFP-positive narrow cells were unchanged from 4 weeks to 4 months. GFP fluorescence was not observed in any region of the epididymal epithelium in 7-month-old GAD67-GFP knock-in mice (data not shown).
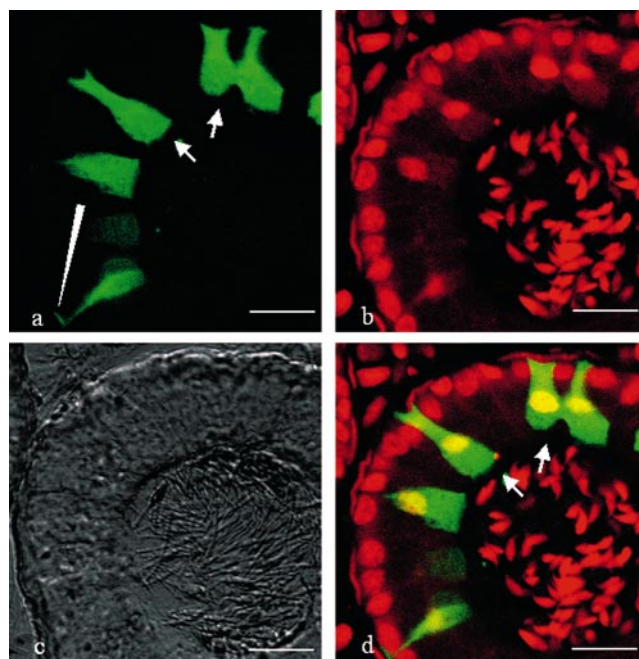
GAD67 and GABA in Narrow Cells

We investigated colocalization of GFP with GAD67 and GABA in 8-week-old GAD67-GFP knock-in mice. Almost all GFP-positive cells were immunopositive for GAD67 (Figure 3) and GABA (Figure 4), and all GAD67- or GABA-positive cells in the epididymis expressed GFP. Epididymal epithelium from wild-type Jcl: ICR mice contained GABA-positive cells, and the morphologic appearance was similar to that in GAD67-GFP knock-in mice (Figure 5).

Analysis of GAD65 and GAD67 mRNAs in Mouse Epididymis

RT-PCR analysis revealed that GAD67 was expressed in GAD67-GFP knock-in mouse epididymis (Figure 6). However, expression of GAD65 mRNA was faint when compared with that of GAD67. In samples from whole brain, mRNAs for 2 isoforms of GAD, GAD65, and GAD67 were expressed.

Analysis of GABA Receptor Subunit mRNAs in Mouse Epididymis

By RT-PCR analysis, GABA$_A$ receptor subunit α1, α2, α5, β1, γ1, and γ3 mRNAs and GABA$_B$ receptor subunit
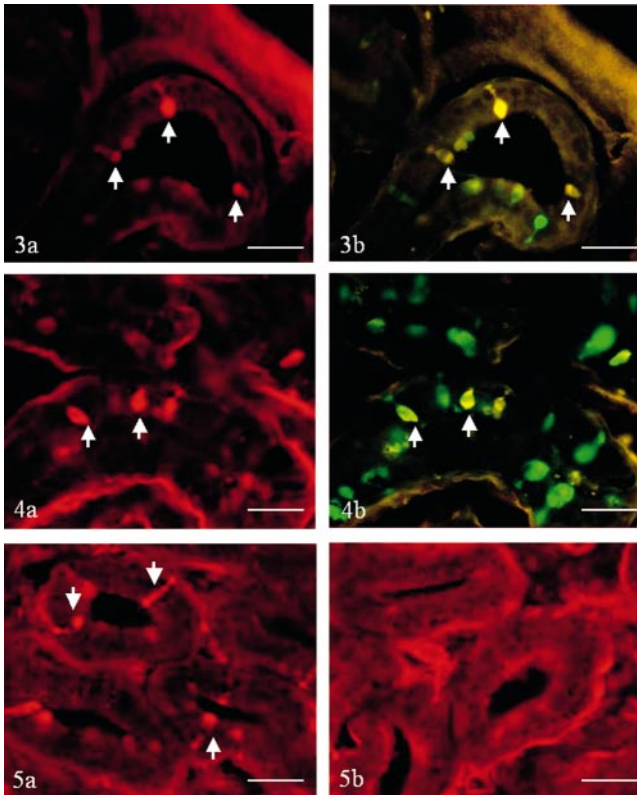
Figure 3. Green fluorescent protein (GFP)–positive cells in the GAD67-GFP knock-in mouse epididymis (8 weeks old) immunostained with rabbit anti-GAD67 antibody. **(a)** GAD67 (red); **(b)** merged image of **Panel a** and GFP fluorescence image. Arrows indicate typical examples of GAD67-positive cells. Scale bar = 25 μm.

Figure 4. Green fluorescent protein (GFP)–positive cells in GAD67-GFP knock-in mouse epididymis (8 weeks old) immunostained with rabbit anti–γ-aminobutyric acid (anti-GABA) antibody. **(a)** GABA (red); **(b)** merged image of **Panel a** and GFP fluorescence image. Arrows indicate typical examples of GABA-positive cells. Scale bar = 25 μm.

Figure 5. Wild-type Jcl:ICR mouse epididymis immunostained with rabbit anti–γ-aminobutyric acid (anti-GABA) antibody. **(a)** GABA (red); **(b)** negative control. GABA immunoreactive cells are present in wild-type mouse caput epididymis, and the cells resemble the green fluorescent protein (GFP)–positive cells in shape (arrows). Scale bar = 25 μm.
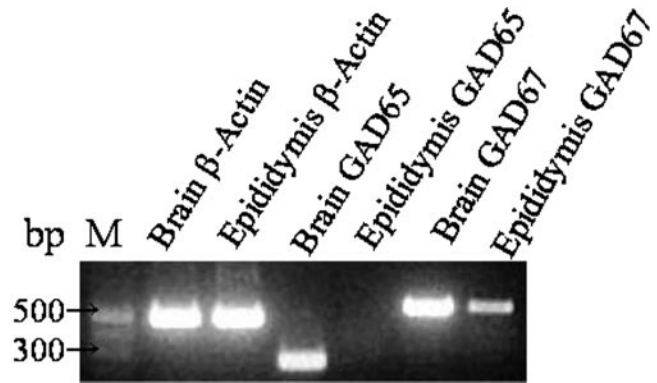

Figure 6. Glutamic acid decarboxylase (GAD) expression in GAD67-GFP knock-in mouse epididymis and brain. Reverse transcription polymerase chain reaction (RT-PCR) results show GAD67 is the predominant isoform in the mouse epididymis.

R1a, R1b, and R2 mRNAs were present in mouse epididymis (Figure 7). Expression of GABA receptor subunit mRNAs in 4 epididymal segments is summarized in Table 2. Among of the $GABA_A$ receptor subunit mRNAs, α2 and β1 subunits were expressed in all 4 epididymal segments, and the level of expression of the β1 subunit was very high, whereas γ1 and γ3 were expressed in only the initial segment and caput epididymis. $GABA_B$ receptor subunit R1b and R2 mRNAs were expressed in all 4 epididymal segments.

Immunohistochemical Analysis of GABA Receptor Subunits in the Epididymis

Immunostaining of epididymides was done for $GABA_A$ receptor subunits α2, β1, and γ1/2/3 and $GABA_B$ receptor subunits R1 and R2 that were detected by RT-PCR. In the initial segment and proximal caput of GAD67-GFP knock-in and wild type Jcl:ICR mice, we did not detect immunoreactivity of $GABA_A$ receptor α2, β1, and γ subunits in the epithelial cells (Figure 8). Spermatozoa, however, produced all $GABA_A$ receptor subunits examined (Figure 8). The $GABA_B$ receptor R1 and R2 subunit immunoreactivities were clearly detected in narrow cells of both GAD67-GFP knock-in and wild-type Jcl:ICR mice (Figure 9). Intense R1 subunit immunoreactivity was seen around nuclei, in the basal feet, and in apical portions of narrow cells in GAD67-GFP knock-in mice (Figure 9a). In the distal caput of the epididymis, moderate R1 subunit immunoreactivity was seen around the nuclei of principal cells (Figure 9a). Intense R1 subunit staining was also detected in narrow cells of wild-type Jcl:ICR mouse epididymis (Figure 9b). Spermatozoa in the epididymal lumen also showed strong R1 subunit immunoreactivity (Figure 9a and b). Localization of $GABA_B$ R2 immunoreactivity differed from that of $GABA_B$ R1. Weak to moderate immunoreactivity for the R2 subunit was seen in the narrow cell cytoplasm (Figure 9c through e), and intense reactivity was seen in the apical portion forming a circular band both in GAD67-GFP knock-in and Jcl:ICR mice (Figure 9d and f). No immunoreactivity was observed in control sections (Figure 9g).

Discussion

The epithelium of the mammalian epididymis contains 5 types of cells: principal, basal, narrow, clear, and halo. The clear (light) cells are located in the cauda epididymis, and halo cells are lymphocytes or macrophages (Hoffer et al, 1973; Dym and Romrell, 1975; Wang and Holstein, 1983). Principal cells are columnar, and the nuclei are located within the basal third of the cells. Basal cells con-
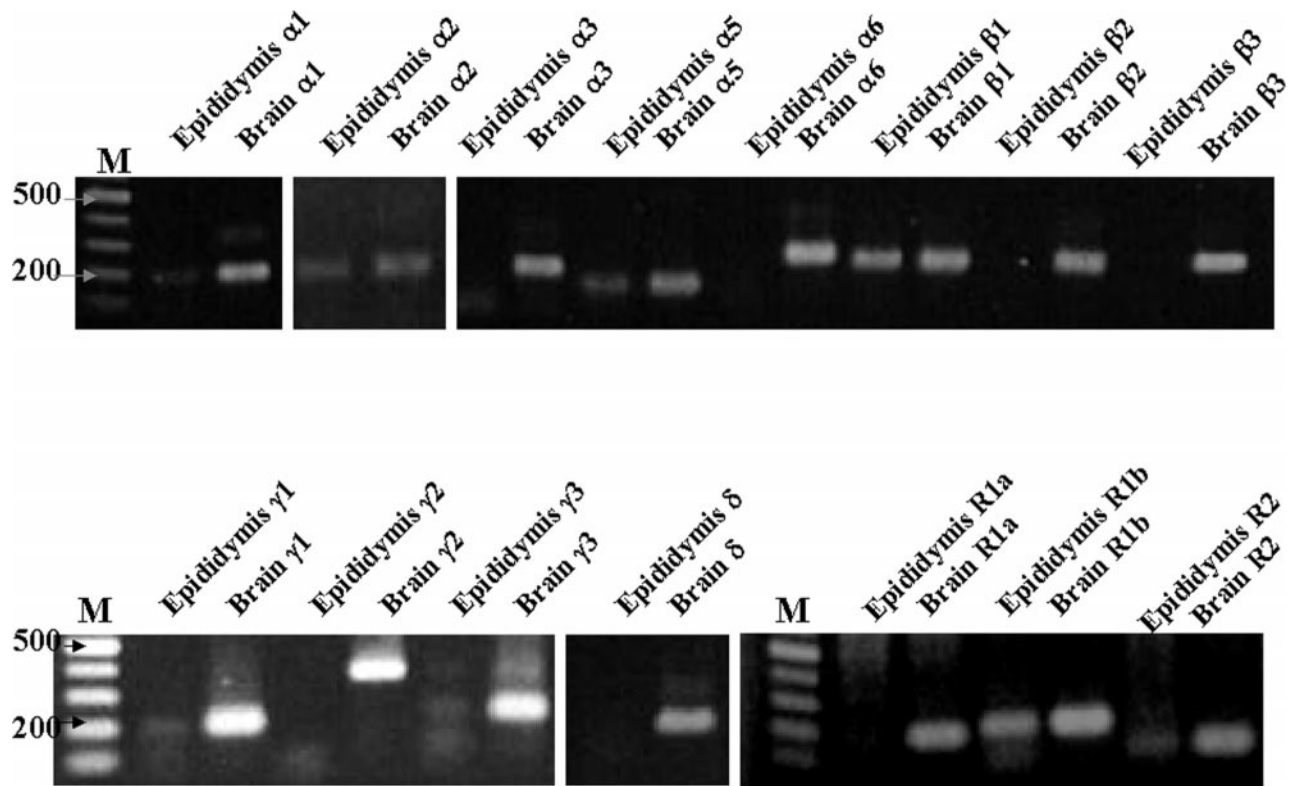
Figure 7.   Reverse transcription polymerase chain reaction (RT-PCR) analysis of γ-aminobutyric acid (GABA)$_A$ and GABA$_B$ receptor subunit mRNAs from GAD67-GFP knock-in mouse total epididymis and brain as a positive control. In the mouse epididymis, α1, α2, α5, β1, γ1, and γ3 GABA$_A$ receptor subunits and R1b and R2 GABA$_B$ receptor subunits are detected.

tain an elongated nucleus parallel to the basal lamina or a prismatic nucleus (Abou-Haila and Fain-Maurel, 1984). The narrow type has a characteristic morphology. Narrow cells contain apically located nuclei and are goblet-shaped with a large basal foot and thin infranuclear region (Abou-Haila and Fain-Maurel, 1984; Adamali and Her-

Table 2. *GABA$_A$ and GABA$_B$ receptor subunit mRNA expression in mouse epididymis**

Subunit	Initial Segment	Caput	Corpus	Cauda	Brain
α1	−	±	−	−	+
α2	+	+	+	+	+
α3	−	−	−	−	−
α5	+	−	−	+	+
α6	−	−	−	−	+
β1	+	+	+	+	+
β2	−	−	−	−	+
β3	−	−	−	−	+
γ1	+	+	−	−	+
γ2	−	−	−	−	+
γ3	+	+	−	−	+
δ	−	−	−	−	+
R1a	−	−	−	±	+
R1b	+	+	+	+	+
R2	+	+	+	+	+

* + indicates positive; −, negative; and ±, faint.

mo, 1996; Hermo and Robaire, 2002). The proximal part of the epididymis contains another cell type: apical cells with an apically located nucleus (Robaire and Hermo, 1988; Hermo and Robaire, 2002). It has been reported that apical and narrow cells are distinct cell types (Adamali and Hermo, 1996; Hermo et al, 2000), and they can be differentiated on the basis of the large basal foot of narrow cells that contacts the basement membrane (Adamali and Hermo, 1996). Furthermore, in this study, GFP-positive cell cytoplasm was stained deeply with toluidine blue, as has been reported elsewhere: the narrow cells can be distinguished readily from apical cells by the staining (Sun and Flickinger, 1980). Therefore, the GFP-positive cells found in the GAD67-GFP knock-in mouse epididymal epithelium were narrow cells. Narrow cells (mitochondrial goblet cells in Abou-Haila and Fain-Maurel, 1984) contain a large number of mitochondria and show high succinate dehydrogenase activity (Abou-Haila and Fain-Maurel, 1984; Martinez-Garcia et al, 1994), suggesting an active tricarboxylic acid cycle that is closely associated with GABA synthesis through the so-called GABA shunt (Watanabe et al, 2002). Although the morphology, histochemistry, distribution, differentiation, and possible function of narrow cells in the mammalian epididymis have been reviewed (Martinez-Garcia et al,

Figure 8. Confocal images of γ-aminobutyric acid (GABA$_A$) receptor α2, β1, and γ subunits immunostaining in the proximal part of the epididymides of wild-type Jcl:ICR mice. **(a)** Immunoreactivity for GABA$_A$ α2 subunit (green) can be seen in spermatozoa, but not in the epithelial cells. **(b)** GABA$_A$ β1 activity (green) is also evident in spermatozoa, but not in the epithelial cells. **(c)** Immunoreactivity for GABA$_A$ γ subunit (green) is present in spermatozoa, but not in the epithelial cells. **(d)** Negative control. Sections were stained with propidium iodide. Scale bars = 50 μm.

Figure 9. Confocal images of γ-aminobutyric acid (GABA)$_B$ receptor subunit immunostaining in the proximal part of epididymides of GAD67-GFP knock-in and wild-type Jcl:ICR mice. **(a)** Immunoreactivity for GABA$_B$ R1 subunit (red) is present in green fluorescent protein (GFP)–positive narrow cells (arrows), in principal cells in the distal caput (arrowhead), and in spermatozoa in the lumen of GAD67-GFP knock-in mouse. **(b)** GABA$_B$ R1 activity (green) is also evident in Jcl:ICR mouse narrow cells (arrow). **(c–f)** GABA$_B$ R2 immunostaining. GFP-positive narrow cells are intensely reactive for the GABA$_B$ R2 subunit (red) in the apical region (arrows in **c, d**). Spermatozoa in the lumen are reactive for GABA$_B$ R2 **(d)**. GABA$_B$ R2 activity (green) is also observed in Jcl:ICR mouse narrow cells (arrows in **e, c**). GABA$_B$ R2 activity in the apical region of narrow cells form a circular band **(d, f)**. **(g)** Negative control. Scale bars = 50 μm.

1994), the function of narrow cells is not fully understood. Proposed functions include uptake of secretions from other cells (Sun and Flickinger, 1980; Cooper et al, 1988), synthesis and secretion of sperm maturation antigen 4 (Feuchter et al, 1987), and acidification of epididymal fluid (Levine and Marsh, 1971; Cohen et al, 1976; Hermo et al, 2000).

In this study, we found that narrow cells in the epithelium of the initial segment and proximal caput of the epididymis expressed GAD67 and GABA. Our RT-PCR data indicate that GAD67 is the predominant GAD isoform in mouse epididymis. Narrow cells of GAD67-GFP knock-in mice exhibited strong GFP fluorescence and GAD67 immunoreactivity. Furthermore, the GFP-positive narrow cells were also positive for GABA. In this study, almost all of the GFP-positive cells expressed GAD67 and GABA, and all GAD67- and GABA-positive cells in the epididymis of GAD67-GFP knock-in mice expressed GFP. In the brain, almost all GFP-positive cells are GABA immunoreactive (Tamamaki et al, 2003; Furuta et al, 2004). These results indicate that the GAD67-GFP knock-in mouse is a powerful tool for detecting and analyzing the GABA-positive cells. We confirmed, by immunostaining wild-type Jcl:ICR mouse epididymis, that expression of GAD67 in the GAD67-GFP knock-in mouse epididymis was not specific to the transgenic
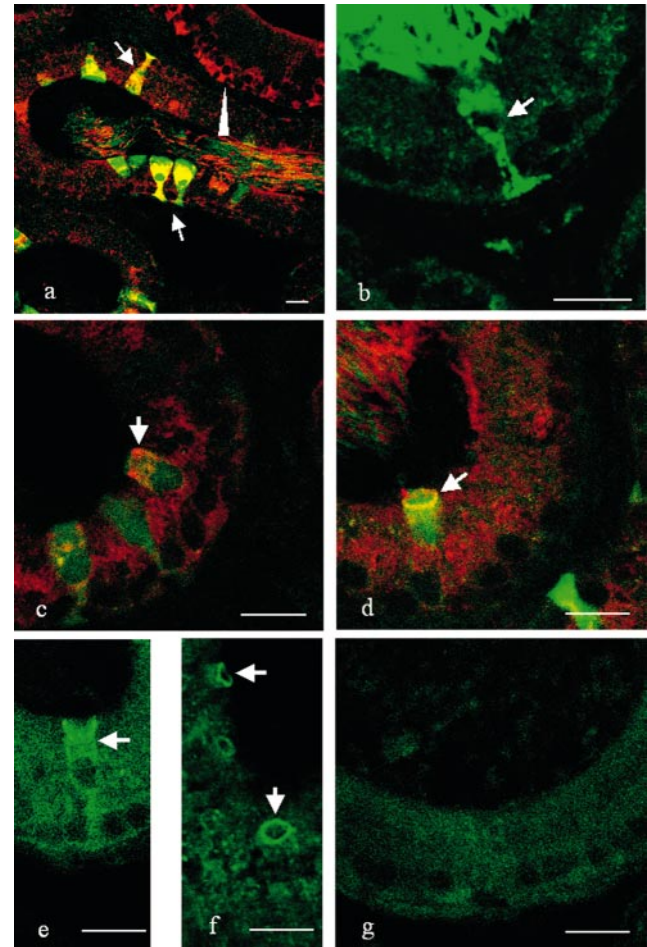
mouse. Anti-GAD67 antibody was also localized in cells in the epididymis of the wild-type Jcl:ICR mouse that have the same morphologic characteristics as narrow cells.

GABA mediates its effects through 2 types of receptors: ionotropic (GABA$_A$ and GABA$_C$) and metabotropic (GABA$_B$; Bowery, 1993; Rabow et al, 1995; Mehta and Ticku, 1999; Couve et al, 2000; Watanabe et al, 2002). GABA$_A$ receptors are pentameric assemblies derived from a combination of at least 16 subunits, and most studies in heterologous expression systems have shown that the functional GABA$_A$ receptor contains at least 1 α, 1 β, and 1 γ subunit (Levitan et al, 1988; Watanabe et al, 2002).

Because RT-PCR revealed that mRNAs of GABA$_A$ receptor subunits α2, β1, γ1, and γ3 were expressed in epididymis, we hypothesized that functional GABA$_A$ receptors might be present in the initial segment and caput epididymis. However, immunohistochemical analysis showed that in the proximal epididymis, epithelial cells exhibited no immunoreactivity for α2, β1, and γ subunits. From the results obtained, it is thought that functional GABA$_A$ receptors are not present in the epithelial cells.

GABA$_B$ receptors are coupled to G proteins, and active functional GABA$_B$ receptors could be a heterodimer of GABA$_B$ R1 and GABA$_B$ R2 subunits (Jones et al, 1998; Kaupmann et al, 1998; White et al, 1998; Kunner et al, 1999; Ng et al, 1999). Both GABA$_B$ receptor subunit mRNAs were expressed in all 4 epididymal segments. The immunohistochemical analysis of this study showed that GABA$_B$ receptor subunit proteins are expressed in GFP-positive narrow cells, indicating colocalization of GABA and its receptors. Thus, GABA could function in narrow cells in an autocrine fashion. In this study, we also showed that GABA$_B$ R1 subunit protein is expressed around the nuclei of principal cells in the distal caput. In contrast to GABA$_B$ R1, GABA$_B$ R2 is not expressed in principal cells. However, the functional significance of GABA$_B$ receptors in the principal cells cannot be ruled out because it has recently been reported that GABA$_B$ R1 might be functional in the absence of GABA$_B$ R2 in the central nervous system (Gassmann et al, 2004; Villemure et al, 2005).

The functional significance of the GABA system in narrow cells and principal cells is not clear. It is known, however, that activation of GABA$_B$ receptors provokes diverse cellular and biochemical responses through modulation of adenylyl cyclase activity or Ca^{2+} or K$^+$ channels (Cunningham and Enna, 1996; Couve et al, 2000; Onali et al, 2003). Recent findings regarding the GABA system in nonneuronal peripheral cells suggest that GABA contributes to cell proliferation via GABA$_B$ receptors in osteoblasts (Fujimori et al, 2002) and in murine embryonal carcinoma-derived ATDC5 cells (Tamayama et al, 2005). Furthermore, in rat testis, GABA might contribute to spermatogenesis via GABA$_B$ receptors (Kanbara et al, 2005).

The effects of GABAergic narrow cells might not be limited to the interaction with epididymal epithelial cells. GABA in narrow cells might have physiological effects on spermatozoa in the lumen. Spermatozoa enter the epididymal tubule from the testis and, during transit, undergo a process of maturation that confers motility and the ability to fertilize an egg (Yanagimachi, 1994; Ong et al, 2000; Cornwall et al, 2002; Hermo and Robaire, 2002). In this study, we showed that the lumen of the proximal epididymis contains strong GFP-positive narrow cells and abundant packaged spermatozoa. These data suggest that there is a significant interaction between GFP-positive cells and spermatozoa in the proximal epididymis, especially in the initial segment and proximal caput. As noted in this study, GABA$_B$ receptors are expressed in spermatozoa (Calogero et al, 1999; He et al, 2001, 2003; Hu et al, 2002a). It has been reported that GABA restores the motility of spermatozoa immobilized by glutamate (Barna and Boldiasár, 1996). In addition, glutamate receptors and transporters have been identified in mouse and human spermatozoa (Hu et al, 2004). Interestingly, glutamate is an important excitatory neurotransmitter, and GABA is a major inhibitory neurotransmitter. Glutamate is catalyzed by GAD to form GABA (Watanabe et al, 2002). Barna and Boldiasár (1996) also reported that GABA causes agglutination of spermatozoa in neutral solution. These findings suggest that GFP-positive narrow cells have functional significance because the lumen of the proximal epididymis contained spermatozoa, and this coincided with the appearance of GFP-positive cells. However, Skandhan (2004) suggested that the epididymis inhibits motility of spermatozoa and that factors are present in the epididymis that make spermatozoa sluggish and inactive. One function of GABA in peripheral nonneuronal tissues is modulation of smooth muscle contraction. GABA inhibits nerve-mediated smooth muscle contraction in the rabbit and human urinary bladder (Santicioli et al, 1984; Chen et al, 1992, 1994) and rat gastroduodenum (Krantis et al, 1998). In contrast, GABA causes contraction of smooth muscle in the female reproductive organs (Erdö et al, 1984; Riesz and Erdö, 1985). Opposing effects of GABA on the contractile activity of the urinary bladder (Maggi et al, 1985) and small intestine (Fargeas et al, 1988) have been reported. In the acrosome reaction, GABA has a biphasic effect (Hu et al, 2002a). This biphasic action is due to the differential interaction of GABA with GABA$_A$ and GABA$_B$ receptors. Opposing effects of GABA or other ligands with GABA receptors are thought to be involved in embryogenesis (Sedlácek, 1988), hormone release (Tapia-Arancibia et al, 1987; Roussel et al, 1988), myenteric cholinergic transmission (Roberts et al, 1993), and embryonic kidney function (Nagata et al, 1994). In this study, α2, β1, γ1, and γ3 subunit mRNAs were expressed in the proximal part of the epididymis. We detected production of all the subunit proteins in spermatozoa in the lumen.

In this study, we provide evidence for GABA system activity in the reproductive tract of male mice. Further studies are needed to clarify the functions of GABA, especially in the narrow cells that express GABA, GAD, and GABA$_B$ receptors.

Acknowledgments

References

Abou-Haila A, Fain-Maurel MA. Regional differences of the proximal part of mouse epididymis: morphological and histochemical characterization. *Anat Rec.* 1984;209:197–208.

Adamali HI, Hermo L. Apical and narrow cells are distinct cell types differing in their structure, distribution, and functions in the adult rat epididymis. *J Androl.* 1996;17:208–222.

Barna J, Boldizsár H. Motility and agglutination of fowl spermatozoa in media of different amino acid content and pH value in vitro. *Acta Vet Hung.* 1996;44:221–232.

Bowery NG. GABA$_B$ receptor pharmacology. *Annu Rev Pharmacol Toxicol.* 1993;33:109–147.

Calogero AE, Burrello N, Ferrara E, Hall J, Fishel S, Rosario D'Agata D. γ-Aminobutyric acid (GABA) A and B receptors mediate the stimulatory effects of GABA on the human sperm acrosome reaction: interaction with progesterone. *Fertil Steril.* 1999;71:930–936.

Calogero AE, Hall J, Fishel S, Green S, Hunter A, D'Agata R. Effects of gamma-aminobutyric acid on human sperm motility and hyperactivation. *Mol Hum Reprod.* 1996;2:733–738.

Chen TF, Doyle PT, Ferguson DR. Inhibitory role of gamma-amino-butyric acid in the rabbit urinary bladder. *Br J Urol.* 1992;69:12–16.

Chen TF, Doyle PT, Ferguson DR. Inhibition in the human urinary bladder by gamma-amino-butyric acid. *Br J Urol.* 1994;73:250–255.

Cohen JP, Hoffer AP, Rosen S. Carbonic anhydrase localization in the epididymis and testis of the rat: histochemical and biochemical analysis. *Biol Reprod.* 1976;14:339–346.

Collier WL, Carlesimo M, Napoleone P, Amenta F. Autoradiographic localization of peripheral GABA$_A$ receptors. In: Erdö SL, ed. *GABA Outside the CNS.* Berlin, Springer-Verlag; 1992:233–247.

Cooper TG, Yeung CH, Bergmann M. Transcytosis in the epididymis studied by local arterial perfusion. *Cell Tissue Res.* 1988;253:631–637.

Cornwall GA, Lareyre JJ, Matusik RJ, Hinton BT, Orgebin-Crist MC. Gene expression and epididymal function. In: Robaire B, Hinton BT, eds. *The Epididymis: From Molecules to Clinical Practice.* New York; Kluwer Academic/Plenum Press; 2002:169–199.

Couve A, Moss SJ, Pangalos MN. GABA$_B$ receptors: a new paradigm in G protein signaling. *Mol Cell Neurosci.* 2000;16:296–312.

Cunningham MD, Enna SJ. Evidence for pharmacologically distinct GABA$_B$ receptor associated with cAMP production in rat brain. *Brain Res.* 1996;720:220–224.

Dym M, Romrell LJ. Intraepithelial lymphocytes in the male reproductive tract of rats and rhesus monkeys. *J Reprod Fertil.* 1975;42:1–7.

Erdö SL, Kiss B. Presence of GABA, glutamate decarboxylase, and GABA transaminase in peripheral tissues: a collection of quantitative data. In: Erdö SL, Bowery NG, eds. *GABAergic Mechanisms in the Mammalian Periphery.* New York: Raven; 1986:5–17.

Erdö SL, Nemet L, Szporny L. The occurrence of GABA in vas deferens, prostate, epididymis, seminal vesicle and testicle of the rat. *Acta Biol Hung.* 1983;34:435–437.

Erdö SL, Riesz E, Karpati E, Szporny L. GABA$_B$ receptor-mediated stimulation of the contractility of isolated rabbit oviduct. *Eur J Pharmacol.* 1984;99:333–336.

Fargeas MJ, Fioramonti J, Bueno L. Central and peripheral action of GABA$_A$ and GABA$_B$ agonists on small intestine motility in rats. *Eur J Pharmacol.* 1988;150:163–169.

Feuchter FA, Green MF, Tabet AJ. Maturation antigen of the mouse sperm flagellum. II. Origin from holocrine cells in the distal caput epididymis. *Anat Rec.* 1987;217:146–153.

Frungieri MB, Gonzalez-Calvar SI, Calandra RS. Influence of photoinhibition on GABA and glutamic acid levels, and on glutamate decarboxylase activity in the testis and epididymis of the golden hamster. *Int J Androl.* 1996;19:171–178.

Fujimori S, Hinoi E, Yoneda Y. Functional GABA$_B$ receptors expressed in cultured calvarial osteoblasts. *Biochem Biophys Res Commun.* 2002;293:1445–1452.

Furuta T, Koyano K, Tomioka R, Yanagawa Y Kaneko T. GABAergic basal forebrain neurons that express receptor for neurokinin B and send axons to the cerebral cortex. *J Comp Neurol.* 2004;473:43–58.

Gassmann M, Shaban H, Vigot R, Sansig G, Haller C, Barbieri S, Humeau Y, Schuler V, Müller M, Kinzel B, Klebs K, Schmutz M, Froestl W, Heid J, Kelly PH, Gentry C, Jaton AL, Van der Putten H, Mombereau C, Lecourtier L, Mosbacher J, Cryan JF, Fritschy JM, Lüthi A, Kaupmann K, Bettler B. Redistribution of GABA$_{B(1)}$ protein and atypical GABA$_B$ responses in GABA$_{B(2)}$-deficient mice. *J Neurosci.* 2004;24:6086–6097.

He XB, Hu JH, Wu Q, Yan YC, Koide SS. Identification of GABA$_B$ receptor in rat testis and sperm. *Biochem Biophys Res Commun.* 2001; 283:243–247.

He XB, Zhang Y, Yan Y, Li Y, Koide SS. Identification of GABA$_B$R2 in rat testis and sperm. *J Reprod Dev.* 2003;49:397–402.

Hermo L, Adamali HI, Andonian S. Immunolocalization of CA II and H+V-ATPase in epithelial cells of the mouse and rat epididymis. *J Androl.* 2000;21:376–391.

Hermo L, Robaire B. Epididymal cell types and their function. In: Robaire B, Hinton BT, eds. *The Epididymis: From Molecules to Clinical Practice.* New York: Kluwer Academic/Plenum Press; 2002:81–102.

Hoffer AP, Hamilton DW, Fawcett DW. The ultrastructure of the principal cells and intraepithelial leucocytes in the initial segment of the rat epididymis. *Anat Rec.* 1973;175:169–201.

Hu JH, He XB, Wu Q, Yan YC, Koide SS. Biphesic effect of GABA on rat sperm acrosome reaction: involvement of GABA(A) and GABA(B) receptors. *Arch Androl.* 2002a;48:369–378.

Hu JH, He XB, Wu Q, Yan YC, Koide SS. Subunit composition of GABAA receptors of rat spermatozoa. *Neurochem Res.* 2002b;27:195–199.

Hu JH, Na Y, Hua MY, Jie J, Fu ZJ, Jian F, He GL. Identification of glutamate receptors and transporters in mouse and human sperm. *J Androl.* 2004;25:140–146.

Jiang B, Kitamura A, Yasuda H, Sohya K, Maruyama A, Yanagawa Y, Obata K, Tsumoto T. Brain-derived neurotrophic factor acutely depress excitatory synaptic transmission to GABAergic neurons in visual cortical slices. *Eur J Neurosci.* 2004;20:709–718.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C. GABA$_B$ receptors function as a heteromeric assembly of the subunits GABA$_{B1}$ and GABA$_{B2}$. *Nature.* 1998;396:674–679.

Kanbara K, Okamoto K, Nomura S, Kaneko T, Shigemoto R, Azuma H, Katsuoka Y, Watanabe M. Cellular localization of GABA and GABA$_B$ receptor subunit proteins during spermiogenesis in rat testis. *J Androl.* 2005;26:35–43.

Kaupmann K, Malitschek B, Schuler V, Heid J, Huggel K, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B. GABA$_B$-receptor subtypes assemble into functional heteromeric complexes. *Nature.* 1998;396:683–687.

Krantis A, Mattar K, Glasgow I. Rat gastroduodenal motility in vivo: interaction of GABA and VIP in control of spontaneous relaxations. *Am J Physiol.* 1998;275:G897–G903.

Kunner R, Kohr G, Grunewald S, Eisenhardt G, Bach A, Kornau HC. Role of heteromer formation in GABA$_B$ receptor function. *Science.* 1999;283:74–77.

Leader A, Munuk GY, Mortimer D. Seminal plasma gamma-aminobutyric acid (GABA) levels in normospernic men. *Clin Invest Med.* 1992; 206:360–364.

Levine N, Marsh DJ. Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tu-

bules, the epididymis and the vas deference in rats. *J Physiol.* 1971;213:557–579.

Levitan ES, Schofield PR, Burt DR, Rhee LM, Wisden W, Kohler M, Fujita N, Rodriguez HF, Stephenson A, Darlison MG. Structural and functional bases for GABA$_A$ receptor heterogeneity. *Nature.* 1988;335:76–79.

Maggi CA, Santicioli P, Meli A. Dual effect of GABA on the contractile activity of the guinea-pig isolated urinary bladder. *J Auton Pharmacol.* 1985;5:131–141.

Martinez-Garcia F, Regadera J, Cobo P, Palacios J, Paniagua R, Nistal M. The apical mitochondria-rich cells of the mammalian epididymis. *Andrologia.* 1994;27:195–206.

Mehta AK, Ticku MK. An update on GABA$_A$ receptors. *Brain Res Brain Res Rev.* 1999;29:196–217.

Nagata K, Hamilton BJ, Carter DB, Narahashi T. Selective effects of dieldrin on the GABAA receptor-channel subunits expressed in human embryonic kidney cells. *Brain Res.* 1994;645:19–26.

Napoleon P, Bronzetti E, Vavallotti C, Amenta F. Predominant epithelial localization of type A gamma-aminobutyric acid receptor sites within rat seminal vesicles and prostate glands. *Pharmacology.* 1990;41:49–56.

Ng GY, Clark J, Coulombe N, Ethier N, Hebert TE, Sullivan R, Kargman S, Chateauneuf A, Tsukamoto N, McDonald T, Whiting P, Merey E, Johnson MP, Liu Q, Kolakowski LFJ, Evans JF, Bonner TI, O'Neill GP. Identification of a GABA$_B$ receptor subunit, gb2, required for functional GABA$_B$ receptor activity. *J Biol Chem.* 1999;274:7607–7610.

Onali P, Mascia FM, Olianas MC. Positive regulation of GABA$_B$ receptors dually coupled to cyclic AMP by the allosteric agent CGP7930. *Eur J Pharmacol.* 2003;471:77–84.

Ong DE, Newcomer ME, Lareyre JJ, Orgebin-Crist MC. Epididymal retinoic acid-binding protein. *Biochem Biophys Acta.* 2000;1482:209–217.

Rabow LE, Russek SJ, Farb DH. From ion currents to genomic analysis: recent advances in GABA$_A$ receptor research. *Synapse.* 1995;21:189–274.

Riesz M, Erdö SL. GABA$_B$ receptors in the rabbit uterus may mediate contractile responses. *Eur J Pharmacol.* 1985;119:199–204.

Ritta MN, Calandra RS. Occurrence of GABA in rat testis and its effect on androgen production. In: Racagni G, Donoso AO, eds. *Advances in Biochemical Psychopharmacology.* Vol 42. *GABA and Endocrine Function.* New York: Raven; 1986:291–297.

Ritta MN, Campos MB, Calandra RS. Coexistence of γ-aminobutyric acid type A and type B receptors in testicular interstitial cells. *J Neurochem.* 1991;42:291–297.

Robaire B, Hermo L. Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* New York: Raven; 1988:999–1080.

Roberts DJ, Hasler WL, Owyang C. GABA mediation of the dual effects of somatostatin on guinea pig ileal myenteric cholinergic transmission. *Am J Physiol.* 1993;264:G953–G960.

Roussel JP, Tapia-Arancibia L, Jourdan J, Astier H. Effect of norfloxacin, a new quinolone of on GABA modulation of TRH-induced TSH release from perifused rat pituitaries. *Acta Endocrinol.* 1988;119:481–487.

Sakai K, Miyazaki J. A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. *Biochem Biophys Res Commun.* 1997;237:318–324.

Santicioli P, Maggi CA, Meli A. GABAB receptor mediated inhibition of field stimulation-induced contractions of rabbit bladder muscle invitro. *J Pharm Pharmacol.* 1984;36:378–381.

Sedlácek J. Changes in the development of spontaneous motility in chick embryos after the chronic administration of GABA. *Physiol Bohemoslov.* 1988;37:307–312.

Skandhan KP. Hypothesis: epididymis inhibits sperm motility inside male reproductive tract. *Med Hypoth.* 2004;62:146–150.

Soghomonian JJ, Martin DL. Two isoforms of glutamate decarboxylase: why? *Trends Pharmacol Sci.* 1998;19:500–505.

Sun EL, Flickinger CJ. Morphological characteristics of cells with apical nuclei in the initial segment of the adult rat epididymis. *Anat Rec.* 1980;196:285–293.

Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T. Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J Comp Neurol.* 2003;467:60–79.

Tamayama T, Maemura K, Kanbara K, Hayasaki H, Yabumoto Y, Yuasa M, Watanabe M. Expression of GABA$_A$ and GABA$_B$ receptors in rat growth plate chondrocytes: activation of the GABA receptors promotes proliferation of mouse chondrogenic ATDC5 cells. *Mol Cell Biochem.* 2005;273:117–126.

Tanaka D, Nakaya Y, Yanagawa Y, Obata K, Murakami F. Multimodal tangential migration of cortical GABAergic neurons independent of GPI-anchored proteins. *Development.* 2003;130:5803–5813.

Tapia-Arancibia L, Roussel JP, Astier H. Evidence for a dual effect of gamma-aminobutyric acid on thyrotropin (TSH)-releasing hormone-induced TSH release from perifused rat pituitaries. *Endocrinology.* 1987;121:980–986.

Villemure JF, Adam L, Bevan NJ, Gearing K, Chenier S, Bouvier M. Sub-cellular distribution of GABA$_B$ receptor homo- and heterodimers. *Biochem J.* 2005;388:47–55.

Wang YP, Holstein AF. Intraepithelial lymphocytes and macrophages in the human epididymis. *Cell Tissue Res.* 1983;233:517–521.

Watanabe M, Maemura K, Kanbara K, Tamayama T, Hayasaki H. GABA and GABA receptors in the central nervous system and other organs. *Int Rev Cytol.* 2002;213:1–47.

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH. Heterodimerization is required for the formation of a functional GABA$_B$ receptor. *Nature.* 1998;396:679–682.

Yanagimachi R. Manmalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* 2nd ed. New York: Raven; 1994:189–317.