

Cellular Localization of GABA and GABA_B Receptor Subunit Proteins During Spermiogenesis in Rat Testis

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ABSTRACT: The GABAergic system, a major inhibitory regulator in the central nervous system, may also play important roles in peripheral nonneuronal tissues and cells. Recent studies showed that GABA_B receptor is expressed in testis and sperm. To understand the role of the GABAergic system in spermiogenesis, we examined cellular localization of GABA and GABA_B receptor subunits in rat spermatids by immunocytochemistry. Immunoreactivity for GABA was detected around acrosomal granules of spermatids during the Golgi and cap phases. GABA_{B(1)} immunoreactivity was observed in the acrosomal vesicle of spermatids in Golgi phase, and during cap phase, this reactivity expanded to the entire region of the acrosome covering the nuclear membrane. The level of reactivity decreased gradually with maturation of spermatids. In contrast, GABA_{B(2)} immunoreactivity was not observed in spermatids during Golgi phase

but was detected in the equatorial region during cap phase. Both GABA immunoreactivity and GABA_{B(2)} immunoreactivity were transferred to the residual cytoplasm during the release of spermatozoa. Electron microscopic immunocytochemistry revealed that, during cap phase, GABA and GABA_{B(1)} were distributed within the whole acrosomal vesicle but not in the acrosomal granule. GABA_{B(2)} immunoreactivity was observed in the narrow space between the inner acrosomal and nuclear membrane and was limited to the equatorial region of the spermatid head. These results indicate that the GABAergic system might be involved in regulation of spermiogenesis.

Key words: Spermatid, immunocytochemistry, acrosome, GABAergic system.

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GABA is known to play important roles as the major inhibitory neurotransmitter in the mammalian central nervous system. GABA binds to ionotropic receptors, GABA_A receptor (GABA_AR) and GABA_CR and/or to a metabotropic receptor, GABA_BR (Bowery, 1993; Mott and Lewis, 1994; Rabow et al, 1995; Mehta and Ticku, 1999; Couve et al, 2000). The GABA_AR is a pentamer composed of various subunits, and the GABA_BR is a heterodimer of GABA_{B(1)} and GABA_{B(2)} (Jones et al, 1998; Kaupmann et al, 1998; White et al, 1998; Kuner et al, 1999). GABA and GABA_AR are found in many nonneuronal peripheral tissues, including both male and female reproductive structures, such as testis (Erdö et al, 1983; Boldizar et al, 1992; Frungieri et al, 1996; Akinci and Schofield, 1999), epididymis (Erdö et al, 1983; Frungieri

et al, 1996), vas deferens (Erdö et al, 1983), ovary (del Rio and Caballero, 1980; Apud et al, 1984; Erdö and László, 1984; Tanaka, 1985; Amenta et al, 1986; Gimeno et al, 1986), oviduct (Erdö et al, 1982; Martin del Rio and Sierra Lopez, 1983; Celotti et al, 1986; Orensanz et al, 1986; Erdö and Wolff, 1990; László et al, 1992), and uterus (Erdö, 1984; Gimeno et al, 1986; Erdö et al, 1989). In peripheral nonneuronal tissues, the putative functions of GABA differ according to the cell type. In the testis, GABA stimulates production of testosterone (Ritta and Calandra, 1986; Ritta et al, 1991). GABA_AR has been identified in the sperm of various mammalian species, indicating that the receptor may mediate the progesterone-initiated acrosome reaction (Wistrom and Meizel, 1993; Roldan et al, 1994; Shi and Roldan, 1995; Turner and Meizel, 1995). Moreover, the interaction between GABA and GABA_AR has also been shown to promote the acrosome reaction of human spermatozoa exocytotic events essential for the fertilization process (Calogero et al, 1996; de las Heras et al, 1997; Shi et al, 1997). Recent studies showed that GABA_BR mRNAs and proteins are also present in testis and sperm (Calogero et al, 1999; He et al, 2001; Hu et al, 2002), indicating that GABA_BR may

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play an important role in mammalian fertilization as an inducer of acrosome reaction (Calogero et al, 1999). Furthermore, expression of glutamate decarboxylase mRNAs, which are GABA synthetic enzymes, has been observed in both round and elongated spermatids. This evidence indicates that GABAergic systems may play modulatory roles in spermatogenesis and mammalian fertilization (Meizel, 1997).

We report here the specific localization of GABA and GABA_BR subunit proteins during rat spermiogenesis as detected by immunocytochemistry at both the light and electron microscopic levels.

Materials and Methods

Animals

Male Sprague-Dawley rats (age, 10 weeks; weight, 220–250 g) were purchased from Clea Japan (Osaka, Japan). The animals were caged in a temperature-controlled room and allowed water and food (CE-2, Clea Japan) ad libitum before being used for experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka Medical College.

Preparation of Tissue Sections for Immunohistochemistry

Rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, Ill; 40 mg/kg body weight), perfused transcardially with Ringer solution, and were then fixed with 50 mL of 4% (wt/vol) paraformaldehyde and 0.05% glutaraldehyde (0.1% glutaraldehyde for GABA) in 0.1 M phosphate buffer (PB; pH 7.4). Testes were dissected and immersed in the same fixatives overnight at 4°C. Testes to be processed for analysis of GABA were fixed in 4% (wt/vol) paraformaldehyde for 4 hours. After brief rinsing with phosphate-buffered saline (PBS), specimens were immersed in 30% sucrose in PBS overnight at 4°C. Specimens were embedded in OCT compound (Miles, Elkhart, Ind), and 7- μ m cuts were made with a cryostat microtome (Leica Microsystems, CM 3056, Nussloch, Germany).

Identification of Stages of Spermiogenesis

The 19 steps in rat spermiogenesis (Clermont, 1960) were identified by hematoxylin staining and periodic acid–Schiff reaction, which have been used to visualize the acrosome. In the present study, we used 4 phases of spermiogenesis: Golgi phase (steps 1–3), cap phase (steps 4–7), acrosome phase (steps 8–14), and maturation phase (steps 15–19).

Immunohistochemistry

We performed immunocytochemistry of GABA_{B(1)}, GABA_{B(2)}, and GABA with goat polyclonal antibody against GABA_{B(1)} subunit (diluted 250 \times ; A19, Santa Cruz Biotechnology Inc, Santa Cruz, Calif) and rabbit polyclonal antibodies against GABA_{B(2)} (diluted 3000 \times ; B32) and GABA (diluted 250 \times ; Chemicon In-

ternational, Temecula, Calif), respectively, as primary antibodies. The specificity of antibody against GABA_{B(1)} (A19) was confirmed in that the antibody reacted with GABA_{B(1)} of mouse and rat origin by Western blotting and immunohistochemistry (Kaupmann et al, 1997). The specificity of antibody against GABA_{B(2)} (B32) was described recently (Li et al, 2001). Using antibodies against GABA_{B(2)} (B32) and GABA (Chemicon), the similar staining patterns were confirmed in the brain (Kulik et al, 2003). Sections were treated in 1% sodium borohydride/PBS for 30 minutes at room temperature (RT). After being washed with PBS, sections were preincubated with normal donkey or goat serum (diluted 50 \times) for 30 minutes at RT and incubated with each primary antibody overnight at 4°C. Sections were then rinsed in PBS and incubated with the appropriate secondary antibody (diluted 300 \times ; Alexa FluorTM 488 donkey anti-goat immunoglobulin G [IgG] or Alexa FluorTM 488 goat anti-rabbit IgG (H+L), Molecular Probes, Eugene, Oreg) for 60 minutes in darkness at RT. Sections were rinsed with PBS and treated with 100 μ g/mL RNase A in PBS for 1 hour at 37°C. Sections were rinsed with PBS and counterstained with 10 μ g/mL propidium iodide (Molecular Probes) in phosphoric and citric buffer for 20 minutes at RT. After several rinses with PBS, immunoreactivity was examined with a confocal laser microscope (Radiance 2000, Bio-Rad Laboratories, Hercules, Calif) equipped with a 488-nm Argon laser.

Electron Microscopic Immunocytochemistry

Sections were preincubated with 20% normal donkey serum in 10 mM PBS (pH 7.4) for 10 minutes at RT, followed by overnight incubation at 4°C with the same primary antibodies used for immunocytochemistry. Sections were then rinsed in PBS and incubated with biotinylated donkey anti-goat IgG (diluted 100 \times ; Chemicon International) or rabbit anti-goat antibody conjugated with 1.4-nm gold particles (Nanoprobes, Stony Brook, NY) for GABA_{B(1)}, biotinylated donkey anti-rabbit IgG (diluted 100 \times ; Chemicon International) or goat anti-rabbit antibody conjugated with 1.4-nm gold particles (Nanoprobes) for GABA_{B(2)}, and biotinylated donkey anti-rabbit IgG (diluted 100 \times ; Chemicon International) for GABA overnight at 4°C. After rinses with PBS, sections were processed for 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining or immunogold staining. For DAB staining, sections were incubated with avidin-biotin-horseradish peroxidase complex (diluted 50 \times ; Vector Laboratories, Burlingame, Calif) for 3 hours at RT, rinsed with PBS, and then incubated with 0.02% DAB in 50 mM Tris-HCl (pH 7.6) containing 0.002% H₂O₂ for 30 minutes at RT. For immunogold staining, sections were washed in 0.1 M PB, postfixated with 1% glutaraldehyde in 0.1 M PB for 10 minutes, washed in 0.1 M PB, washed in distilled water, and reacted with an HQ Silver Enhancement Kit (Nanoprobes). For both protocols, sections were rinsed in distilled water and then washed in 0.1 M PB. Sections were treated with 1% OsO₄ in 0.1 M PB for 40 minutes. Sections were rinsed in distilled water and counterstained with 1% (wt/vol) uranyl acetate for 30 minutes. Sections were then dehydrated through a graded ethanol series and flat-embedded in Epoxy-resin (Luveak; Nacalai Tesque, Kyoto, Japan). Ultrathin sections were prepared on an ultramicrotome (Reichert-Nissei

Ultracut S; Leica, Vienna, Austria) and observed under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

Results

Immunohistochemistry

During Golgi phase, GABA_{B1} immunoreactivity was observed in the acrosomal vesicle of spermatids (Figure 1A and D; Figure 2A). During cap phase, GABA_{B1} was present in the entire area of the acrosome covering the nuclear membrane (Figure 1B and E; Figure 2B). The distribution patterns of GABA_{B1} immunoreactivity differed slightly between the early and late stages of cap phase. In the early stage of cap phase, intense reactivity was observed in the apical segment of the acrosome, but this apical segment staining decreased in the late stage (Figure 1B). During the acrosome phase, GABA_{B1} immunoreactivity was observed in the equatorial and principal regions of the acrosome of elongated spermatids (Figure 1C and F; Figure 2C). Immunoreactivity in the ventral region was more intense than that in the dorsal region. During maturation, staining intensity decreased gradually and finally disappeared (Figure 2D).

Localization of GABA_{B2} immunoreactivity was different from that of GABA_{B1}. GABA_{B2} immunoreactivity was not detected during Golgi phase (Figure 2E). During cap phase, GABA_{B2} staining first appeared in the equatorial region of the spermatid head and formed a circular band (Figure 2F). During the acrosome phase, GABA_{B2} immunoreactivity was observed in the equatorial and proximal regions of the cytoplasmic lobe (Figure 2G). During maturation phase, GABA_{B2} immunoreactivity was observed throughout residual cytoplasm at the time of the release of spermatozoa (Figure 2H).

GABA immunoreactivity was visible around the rims of the acrosomal granules during Golgi phase (Figure 2I). During cap phase, GABA immunoreactivity was present exclusively in the equatorial region of the spermatid head in a spherical shape (Figure 2J). During acrosome phase, GABA immunoreactivity was visible at the dorsal part of the acrosome (Figure 2K). From acrosome to maturation phase, GABA immunoreactivity was divided into two regions. Reactivity was transferred to the anterior acrosomal segment and to the residual cytoplasm (Figure 2L). However, the level of reactivity of the anterior segment tended to decrease during the maturation process. For negative control, sections were incubated with nonimmune sera from the same species as the primary antibody. Negative controls showed no specific staining.

Electron Microscopic Immunocytochemistry

The distribution patterns of GABA_{B1}, GABA_{B2}, and GABA during spermiogenesis were observed in greater

detail by electron microscopic immunocytochemistry. During Golgi phase, GABA_{B1} immunoreactivity was observed over the surface of the acrosomal granule within the outer acrosomal membrane (Figure 3A). In the early and late stages of cap phase, the reactivity was distributed broadly within the whole acrosomal vesicle, excluding the acrosomal granule (Figure 3B). Immunogold particles did not appear to associate with the inner or outer acrosomal membrane (Figure 4A). The level of the reactivity in the acrosomal vesicle decreased gradually during the maturation process (Figure 3C) and finally disappeared during maturation phase (Figure 3D). GABA_{B2} immunoreactivity was not detected during Golgi phase (Figure 3E). GABA_{B2} immunoreactivity was localized in the equatorial region of the spermatid head during cap phase (Figure 3F). Furthermore, immunogold particles were observed in the narrow space between the inner acrosomal membrane and the nuclear membrane in the equatorial region (Figure 4B). During the acrosome phase, ladder-like staining was observed in the cytoplasmic lobe as the manchette formation progressed (Figure 3G). During maturation phase, low-level staining was observed in the equatorial region of the spermatid head, and a somewhat higher intensity of staining was observed in the residual cytoplasm (Figure 3H). GABA immunoreactivity was distributed within the whole acrosomal vesicle, with the exception of the acrosomal granule (Figure 5). No staining was observed in testicular tissue incubated with normal serum.

Discussion

In the present study, we examined localization of GABA and GABA_B subunits in spermatids during spermiogenesis by immunocytochemistry and found that, during Golgi phase, GABA_{B1} is localized only in the acrosomal vesicle. Electron microscopic immunocytochemistry clarified that GABA_{B1} is present specifically across the periphery of the acrosome granule. During cap phase, GABA_{B1} is expressed across the entire acrosome, with the exception of the acrosomal granule. In contrast to GABA_{B1}, GABA_{B2} is not expressed during the Golgi phase and first appears during cap phase along the equatorial region of the spermatid head and forms a ring-shaped band. Immunoelectron microscopy revealed that GABA_{B2} is localized in the narrow space between the inner acrosomal membrane and the nuclear membrane in the equatorial region. It has been suggested that GABA_BR function requires formation of heterodimers composed of both GABA_{B1} and GABA_{B2} (Jones et al, 1998; Kaupmann et al, 1998; White et al, 1998; Kuner et al, 1999). Each GABA_{B1} and GABA_{B2} contains a large extracellular N (amino)-terminal domain, 7 hydrophobic membrane-spanning domains, and an intracellular C (car-

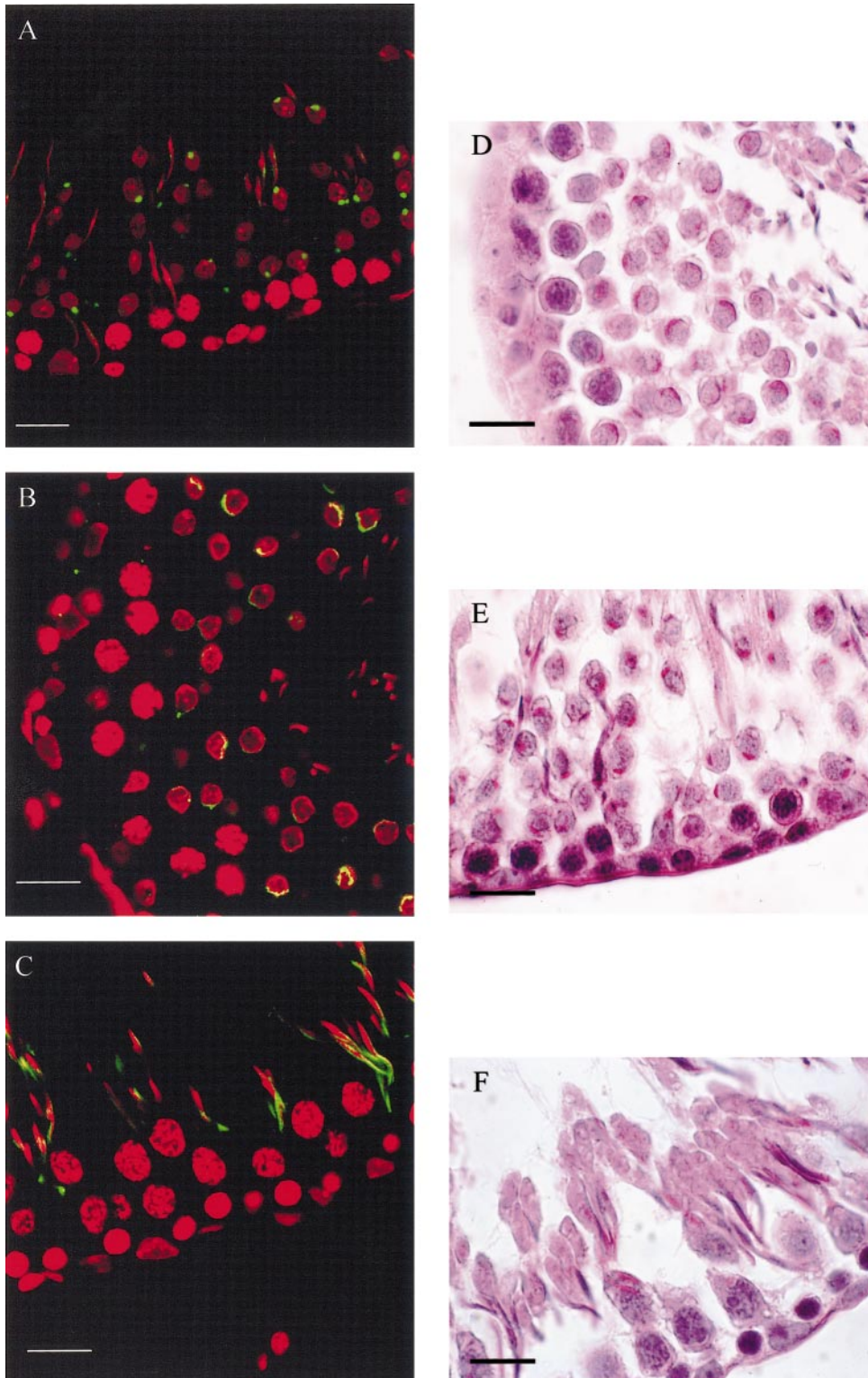


Figure 1. Laser scanning microscopic images in rat seminiferous tubules (A–C) GABA_{B(1)} immunoreactivity is visible as green fluorescence. Cell nuclei are stained with PI (red). The periodic acid–Schiff (PAS) reaction has been used to visualize the acrosome (D–F). Stages of the cycle of seminiferous epithelium were determined from PAS and hematoxylin staining patterns (A and D, Stages II–III; B and E, Stages VI–VII; C and F, Stages XII–XIII). Scale bar = 10 μ m.

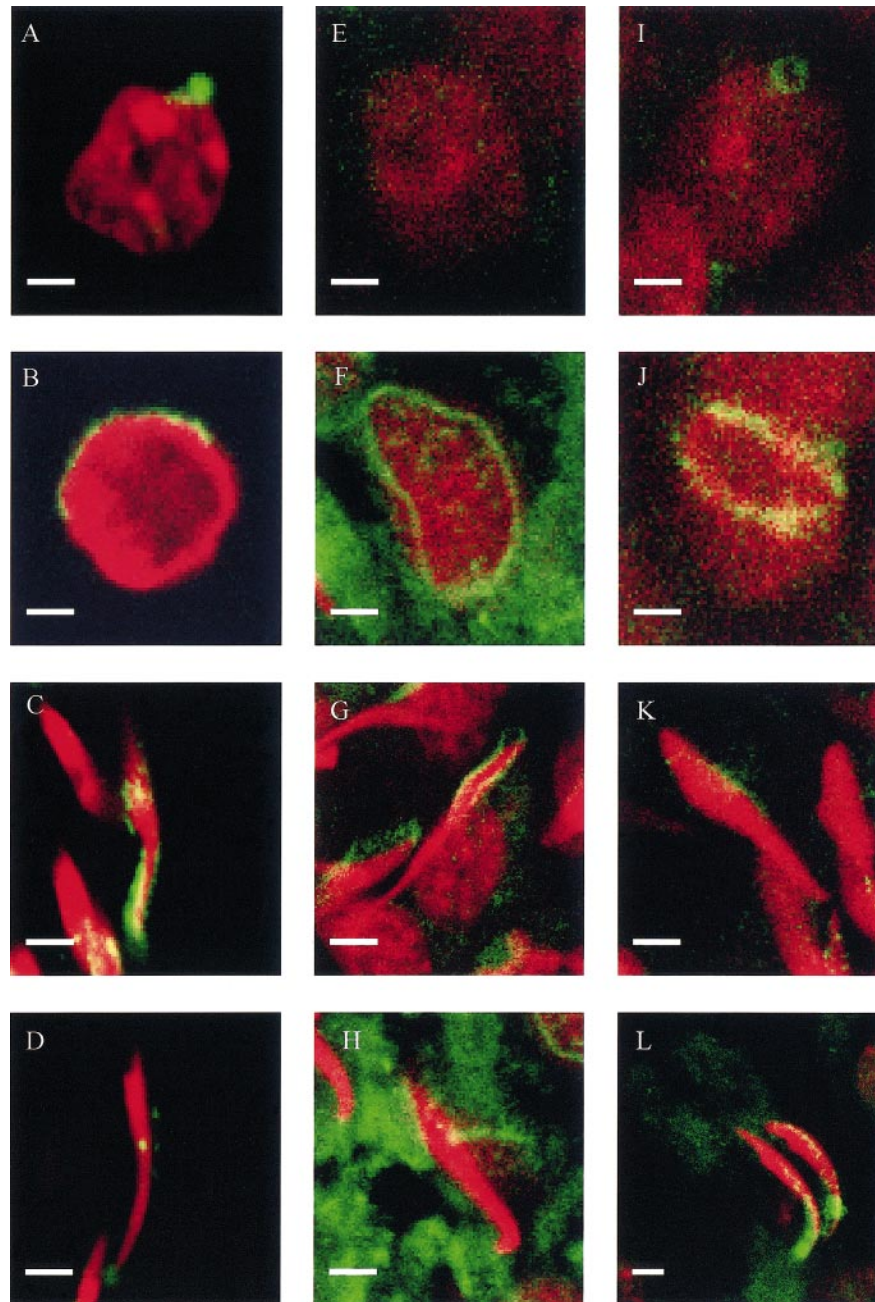


Figure 2. A series of laser scanning microscopic images by indirect immunofluorescence. Cellular localization of GABA_{B(1)} (A–D), GABA_{B(2)} (E–H), and GABA (I–L) in each spermatid phase during spermiogenesis. Golgi phase (A, E, I), cap phase (B, F, J), acrosome phase (C, G, K), and maturation phase (D, H, L). Immunoreactivity of each antibody is visible as green fluorescence. Nuclei of all cells are seen as red fluorescence due to PI staining. During Golgi and cap phases, GABA_{B(1)} was localized in the acrosomal vesicle (A, B). In contrast, GABA_{B(2)} was not expressed during the Golgi phase (E) and first appeared in the equatorial region during cap phase (F). GABA immunoreactivity was present around the rims of the acrosomal granules during Golgi phase (I) and exclusively in the equatorial region of the spermatid head during cap phase (J). During the maturation process, staining intensity of GABA_{B(1)} tended to decrease (D). Scale bar = 1 μ m.

boxy)-terminal domain that contains a coiled-coil domain that interacts with the two subunits (Kammerer et al, 1999; Kuner et al, 1999). The polyclonal anti-GABA_{B(1)} antibody used in the present study recognizes the N-terminus, and the polyclonal anti-GABA_{B(2)} antibody recognizes the C-terminus. The N-terminus of the GABA_{B(1)}

contains the GABA binding site (Jones et al, 2000; Calver et al, 2001; Galvez et al, 2001). The immunoreactivities of GABA and GABA_{B(1)} were present inside the acrosome, whereas that of GABA_{B(2)} was located outside the acrosome. Therefore, our results indicate that, during the late stage of cap phase, two GABA_BR subunits may form

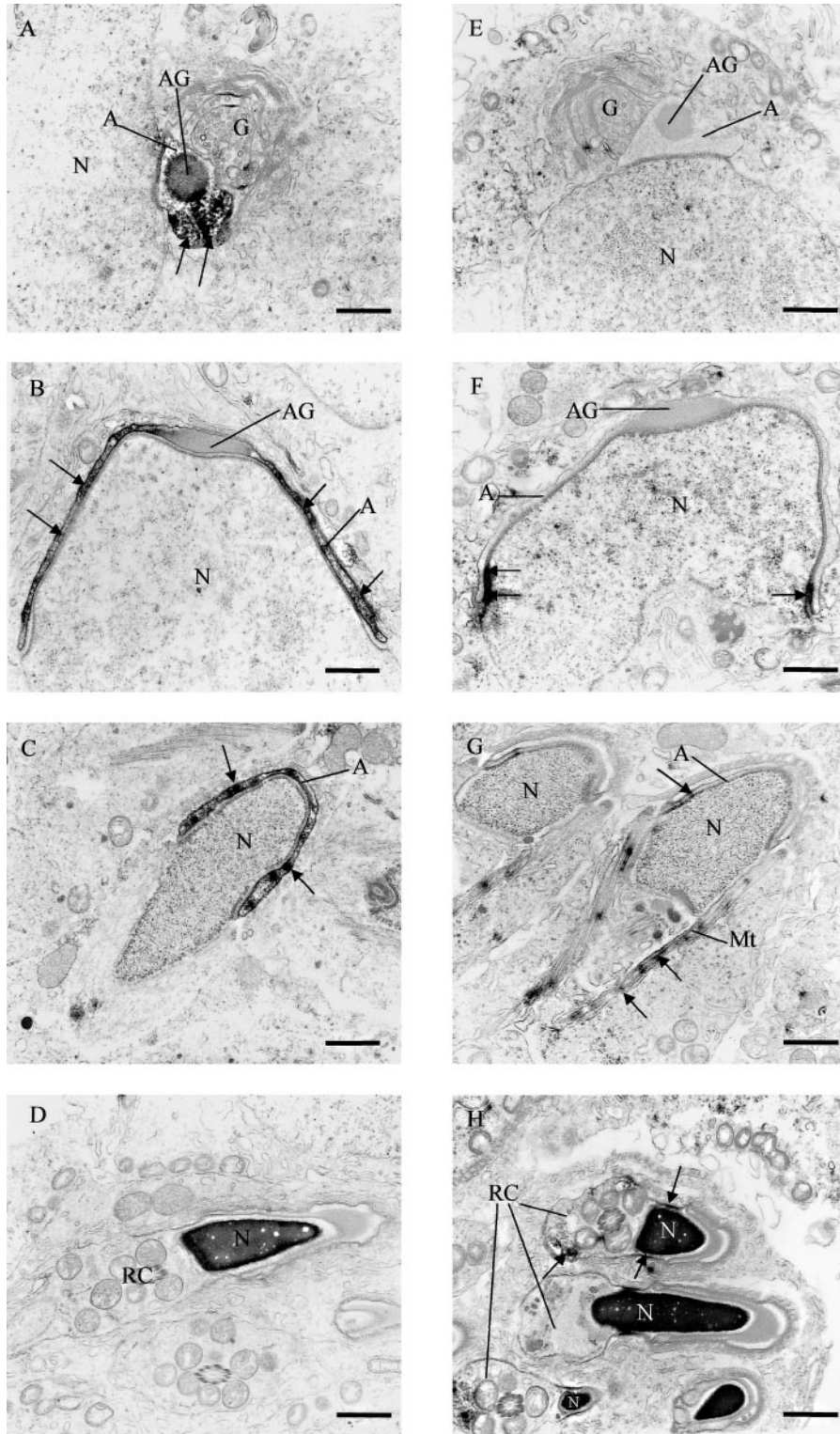


Figure 3. Immunoelectron micrographs of $GABA_{B(1)}$ (A–D) and $GABA_{B(2)}$ (E–H) in each spermatid phase during spermiogenesis by DAB staining. Golgi phase (A, E), late stage of cap phase (B, F), acrosome phase (C, G), and maturation phase (D, H). In late stage of spermiogenesis, the nucleus is highly condensed. A indicates acrosome; AG, acrosomal granule; G, Golgi apparatus; Mt, manchette; N, nucleus; and RC, residual cytoplasm. Arrows indicate immunoreactivity. Scale bar = 1 μ m.

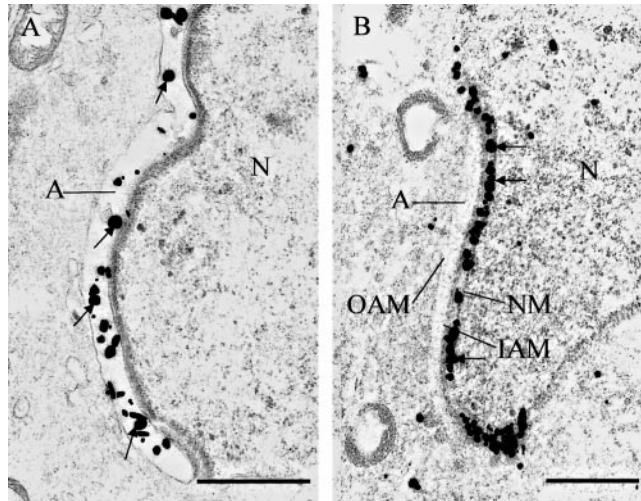


Figure 4. Immunoelectron micrographs in the late stage of cap phase with GABA_{B(1)} (A) and GABA_{B(2)} (B) antibodies by immunogold staining. Arrows indicate gold particles. GABA_{B(1)} immunoreactivity was distributed broadly within the whole acrosomal vesicle. The immunogold particles did not appear to associate with the inner or outer acrosomal membrane (A). The immunogold particles of GABA_{B(2)} were observed in narrow space between the inner acrosomal membrane and the nuclear membrane in the equatorial region (B). A indicates acrosome; IAM, inner acrosomal membrane; N, nucleus; NM, nuclear membrane; and OAM, outer acrosomal membrane. Scale bar = 1 μ m.

functional heterodimers in the inner acrosomal membrane and that GABA, which is located inside the acrosome, may be able to bind these receptors. However, GABA_{B(1)} immunoreactivity was first observed in the acrosomal vesicle of spermatids during Golgi phase. On the other hand, GABA_{B(2)} first appeared during cap phase. This means that until the cap phase, GABA_{B(1)} and GABA_{B(2)} are not co-localized. In addition, our electron microscopic immunocytochemistry revealed that GABA_{B(1)} immunoreactivity was detected even in the acrosome away from the equatorial region at late stage of cap phase. If GABA_BR needs to form the heterodimer, the GABA_{B(1)} does not play a functional role. Recently, we have learned that GABA_{B(1)} might be functional in the absence of GABA_{B(2)} (Gassmann et al, 2004; Villemure et al, 2005). Therefore, the possibility that GABA_{B(1)} may be functional as homodimeric GABA_BR and/or heterodimer with unknown protein cannot be ruled out. It has been suggested that some proteins localized to the equatorial region of the spermatid head regulate the manchette function, which is involved in sperm nuclear shaping or cytoplasmic reorganization (Russel et al, 1991; Moreno and Schatten, 2000; Toshimori et al, 2001). GABA and GABA_BR subunits are co-localized in the equatorial region during the late stage of cap phase, although the level of immunoreactivity for GABA and GABA_BR subunits tends to decrease gradually and finally disappear as spermatids mature. These results indicate that GABA and GABA_BR may have roles in the drastic morphological change of sper-

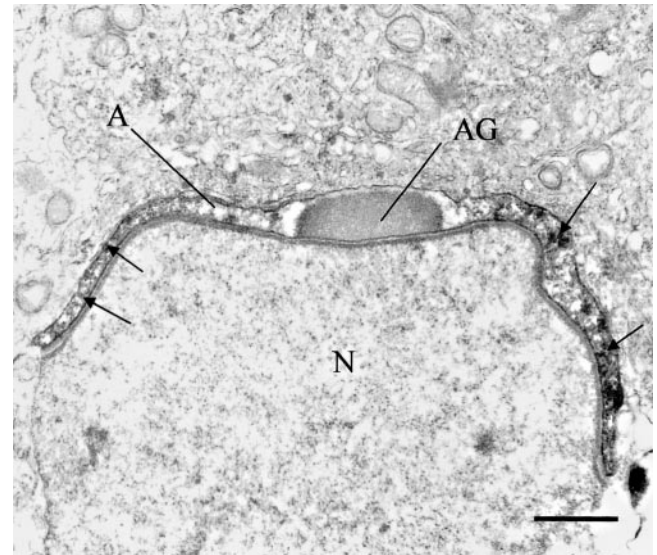


Figure 5. Immunoelectron micrograph in the late stage of cap phase with anti-GABA antibody by DAB staining. Arrows indicate immunoreactivity. GABA immunoreactivity was observed within the whole acrosomal vesicle. A indicates acrosome; AG, acrosomal granule; and N, nucleus. Scale bar = 1 μ m.

matids from round to elongated through GABA_BR signaling pathways such as inhibition of adenylyl cyclase, inactivation of voltage-dependent calcium channels, and activation of potassium channels (Kerr and Ong, 1995; Bettler et al, 1998; Bowery and Enna, 2000; Couve et al, 2000).

During the process of elongation of spermatids, GABA_{B(1)} is expressed in the equatorial and principal regions of the acrosome. Levels of GABA_{B(1)} gradually decrease and finally disappear during maturation. In the present study, after GABA_{B(2)} localized to the equatorial region, we observed ladder-like staining as the manchette formation progressed. GABA_{B(2)} is then released to the residual cytoplasm during maturation phase. GABA gradually translocates to the anterior acrosomal segment and localizes in the residual cytoplasm at the final step of spermiogenesis. A similar phenomenon has been reported for an acrosomal structural matrix protein, acrin 2, in which excess acrosomal contents are eliminated via residual bodies by Sertoli cells (Yoshinaga et al, 2001). Reduction of acrosomal contents is necessary for spermatozoa to move efficiently toward the ova in the female reproductive tract. These findings indicate that excess GABA and GABA_BR, which are involved in maturation of spermatids, are eliminated via residual bodies.

Taken together, these data support the hypothesis of Geigerseder et al. (2003), who asserted that GABA and GABA_BR subunits, which are expressed in a distinct stage-specific manner during spermiogenesis, may play important roles in spermiogenesis. Although the physiological role of the GABAergic system in spermiogenesis

is not yet known, further studies with GABA_BR knockout mice or cultured cells from wild-type mice will clarify the precise mechanisms and functions of the GABAergic system in spermiogenesis.

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