

The cellular expression of GABA_A receptor α 1 subunit during spermatogenesis in the mouse testis

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Summary. GABA_A receptors are pentamers in structure and are mainly composed of α , β and γ subunits. These receptors are known to function as chloride channels. We observed α 5, β 1 and γ 3 subunit immunoreactivity in the mouse testes, specifically in the cytoplasm surrounding the nucleus in the spermatocytes and spermatids. In the current study, α 1 subunit immunoreactivity was located in the nucleus of spermatogonia, spermatocytes and round spermatids. Immunoelectron microscopy revealed that the α 1 subunit was localized within the nucleus of pachytene and diplotene spermatocytes in the area of condensed chromatin rather than extended chromatin. Protein sequence analysis revealed that the α 1 subunit included DM DNA binding domains that were related to transcription factors involved in testicular differentiation in adult mice. These findings suggest that the α 1 subunit may undertake a gene transcription function during the maturation of germ cells. α 1 immunoreactivity was also detected within the mitochondria of spermatocytes and in the acrosome of round and elongated spermatids. Although the precise physiological role of the GABA_A receptor α 1 subunit in mitochondria remains unknown, we hypothesize that its function in the acrosome may be related to the acrosome reaction during fertilization or during spermatogenesis.

Key words: Electron microscopy, GABAergic system, Male reproductive system, Polymerase chain reaction

Introduction

γ -Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the adult mammalian brain and is also detected in numerous peripheral tissues (Watanabe et al, 2002). GABA exerts its effects via two types of receptors, termed the ionotropic GABA_A and GABA_C receptors, and the metabotropic G protein-coupled GABA_B receptor. GABA_A receptors are pentamers in structure and are comprised of a combination of various subunits including α , β , γ and δ subunits (McKernan and Whiting, 1996; Barnard et al., 1998; Costa, 1998; Mehta and Ticku, 1999; Watanabe et al., 2002). As a result of the numerous possible combinations of these subunits, the GABA_A receptors represent a diverse family of receptors. Heterogeneity in GABA_A receptor structures may also reflect functional variation in different cell types. The GABAergic system is thought to play important roles in proliferation, migration, differentiation and the promotion of cell survival in neuronal tissues and in numerous peripheral non-neuronal tissues (Fiszmman and Schousboe, 2004; Magnaghi et al., 2004; Wang et al., 2005; Tamayama et al., 2005; Xiouri and Papazafiri, 2006). It has been reported that embryonic stem cells and peripheral neural crest cells produce GABA, and that this GABA regulates stem cell proliferation through the phosphatidylinositol-3-OH kinase-related family of kinases and H2AX following the activation of GABA_A receptors (Andäng et al., 2008).

It is generally considered that GABA_A receptors function merely as pentamer receptors. Recent data, however, have suggested that the α 1 subunit alone plays an important role in gene transcription events in the NIH3T3 fibroblast cell line (Vaknin and Hann, 2006).

The expression of the α 1 subunit is known to be regulated by c-myc, which in turn functions as a transcriptional regulator. Up-regulation of the α 1 subunit correlates with a down-regulation in c-myc protein levels, resulting in an increase in cell proliferation. In contrast, over-expression of the α 1 subunit induces apoptosis. This finding may therefore also indicate that the α 1 subunit plays a modulator role in cell proliferation and cell survival.

Male germ cell differentiation is based on differential gene expression. The spermatogenic cell-specific transcription associated with chromatin structure is known to be essential for the differentiation processes that occur during spermatogenesis (McCarrey et al., 2005; Zheng et al., 2008). In the male reproductive system, the interaction between GABA and GABA_A-like receptors has also been shown to induce the acrosome reaction in the human spermatozoa, an exocytotic event essential for fertilization (Calogero et al., 1996; de las Heras et al., 1997; Shi et al., 1997). GABA receptors and GAD67 mRNAs have been identified in the rat testis and sperm using reverse transcription-polymerase chain reaction (RT-PCR) during the early stages of spermatogenesis (Tillakaratne et al., 1995; Akinci and Schofield, 1999). Our recent immunoelectron microscopy study revealed that GABA localizes within the entire acrosomal vesicle with the exception of the acrosomal granule during spermiogenesis. A similar distribution was also observed for the GABA_{B1} receptor R1 subunit, while the R2 subunit was localized to the narrow space between the inner acrosome and nuclear membrane (Kanbara et al., 2005). These findings indicate that the GABAergic systems may play modulatory roles in spermiogenesis. However, the precise physiological role of the GABA_A receptor in spermiogenesis remains unknown. In the current study, we investigated GABA_A receptor subunit mRNA expression by RT-PCR, and identified the specific localization of the GABA_A receptor α 1 subunit during spermatogenesis using confocal and electron immunocytochemistry.

Materials and methods

Animals

7- to 8-week-old BALB/c mice were purchased from Clea Japan (Osaka, Japan). The animals were housed in a temperature-controlled room with water and food (CE-2, Clea Japan) supplied *ad libitum*. All experimental protocols were reviewed and approved in advance by the Ethics Review Committee for Animal Experimentation of Osaka Medical College.

RNA isolation and RT-PCR of spermatogenic cells

BALB/c mice were anesthetized with diethylether and sodium pentobarbital (50mg/ kg, i.p.; Abbott

Laboratories, North Chicago, IL, USA) and the testes rapidly excised (n=10). In order to isolate spermatogenic cells, seminiferous tubules were incubated at 33°C in 1 mg/ml collagenase type I (Sigma Chemical Co., St Louis, USA) and 0.4 mg/ml DNase type I (Sigma Chemical Co.) in PBS for 30 min. Total RNA of spermatogenic cells was then extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and first-strand cDNA synthesized using Omniscript Reverse Transcriptase (Qiagen GmbH) according to the manufacturer's instructions. Briefly, total RNA from 1 μ g of tissue was diluted in 12 μ l RNase free water and incubated in a 20 μ l reaction mixture containing 1 μ M oligo-d (T)12–18 primer, 10 units RNase inhibitor, 0.5 mM of each dNTP and 4 units Omniscript reverse transcriptase for 60 min at 37°C. PCR was then performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster, CA, USA) as follows. The reaction mixture (25 μ l) consisted of 1x GoTaq Green Master Mix reaction buffer (pH 8.5) containing 400 μ M dNTP and 3 mM MgCl₂, (Promega, Madison, WI 53711-5399, USA), 2 μ l cDNA solution and 0.2 μ M of each primer. The sequences for the GABA_A α 1-6, β 1-3, γ 1-3 and δ subunit primer pairs are presented as follows (Abe et al., 2005; Tamayama et al., 2005). The primer sequences were 5'-CAGCAAGAACTGTCTTTGGAG-3' and 5'-GCATACCCTCTCTTG GTGAA-3' for α 1, 5'-TCAGTGCTCGAAATTCCT-3' and 5'-GTATCATGACGGAGCCTTTC-3' for α 2, 5'-ATGTCGTCATGACAACCA-3' and 5'-GTTTCTGGCAC TGATACTCAAG-3' for α 3, 5'-GTCTCCCTCTCAAC AACCT-3' and 5'-GTAGAGAAGCGTGCCATCA-3' for α 5, 5'-TGGGCAAACAGTTTCTAGTG-3' and 5'-AGCAGTGTGCTTAAGGTG-3' for α 6, 5'-TGGCTATAC CACGGATGAC-3' and 5'-AGTGTGGAAGGCATG TAGG-3' for β 1, 5'-GGCTACTTCATCCTGCAGA-3' and 5'-GAAGGGCCATAAAGACGAAG-3' for β 2, 5'-GCCATCGACATGTACCTGA-3' and 5'-GAATTCCT GGTGTCACCAAC-3' for β 3, 5'-CGGATGGGCTA TTTCACGA-3' and 5'-CATTGCTGTCACGTAAGA AAC-3' for γ 1, 5'-ACTTCCCAATGGATGAACAC-3' and 5'-TGTCATGGTCAGGACAGTAG-3' for γ 2, 5'-TCAAGCTGTGCGAAAGCCA-3' and 5'-GTCTTTG CCATCCAGACT-3' for γ 3, 5'-GCCAGAGTATC TCTAGGCA-3' and 5'-CGTTCCTCACATCCATCTC-3' for δ , and 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGATTTTC-3' for β -actin. The amplification conditions consisted of a 2 min pre-incubation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, and a single 5-min incubation at 72°C. The PCR products were separated on 1.5% agarose gels. Gels were then stained for 20 min in 0.1% ethidium bromide, visualized by UV transillumination and documented on black and white instant film. We used a brain as positive control. The PCR for β -actin was performed by the brain and testis samples without a reverse transcription step as negative control.

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Preparation of tissue sections for immunohistochemistry

BALB/c mice were anesthetized with an intraperitoneal injection of sodium pentobarbital, perfused transcardially with Ringer's solution and fixed with 50 ml of 4% (w/v) paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Testes were then dissected and immersed in the same fixative overnight at 4°C. After brief rinsing in phosphate-buffered saline (PBS), specimens were immersed in 30% sucrose in PBS overnight at 4°C. Specimens were then embedded in OCT compound (Miles, Elkhart, IN, USA) and sectioned at 7 μ m using a cryostat (Leica Microsystems, CM 3056, Nussloch, Germany).

Confocal immunomicroscopy

We performed immunocytochemistry analysis on the GABA_A receptor subunits α 1, α 5, β 1-3 and γ 3 that were detected by RT-PCR using goat polyclonal antibodies (diluted 250x; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (Hayasaki et al., 2006). After washing with PBS, cryosections were pre-incubated with 3% normal donkey serum for 30 min at room temperature (RT) and incubated with each primary antibody overnight at 4°C. Sections were then rinsed in PBS and incubated with the Alexa FluorTM 488 donkey anti-goat IgG antibody (diluted 300x; Molecular Probes, Eugene, OR, USA) for 60 min in darkness at RT. Sections were then rinsed with PBS and treated with 100 μ g/ml RNase A (Sigma, St Louis, Mo, USA) in PBS for 1 h at 37°C. Sections were subsequently rinsed with PBS and counterstained with 10 μ g/ml propidium iodide (PI, Molecular Probes) in phosphoric and citric buffer for 3 min at RT. After several rinses with PBS, immunoreactivity was examined using a confocal laser microscope (LSM510, Co., Ltd, Oberkochen, Germany) equipped with a 488-nm Argon laser. Stages of the cycle of seminiferous epithelium were determined from PAS and hematoxylin staining patterns (data not shown, Kanbara et al., 2005). For a negative control, sections were incubated with non-immune sera from the same species as the primary antibody. Negative controls revealed no specific staining.

Immunoelectron microscopy

Cryosections were pre-incubated with 20% normal donkey serum in 10 mM PBS (pH 7.4) for 10 min at RT, followed by overnight incubation at 4°C with the α 1 subunit primary antibody used for the confocal immunomicroscopy. Sections were then rinsed in PBS and incubated with biotinylated donkey anti-goat IgG (diluted 100x; Chemicon International, Temecula, CA, USA) overnight at 4°C. After rinsing with PBS, sections were processed for 3, 3'-diaminobenzidine tetrahydrochloride (DAB) staining. Briefly, the sections were incubated with avidin-biotin-horseradish peroxidase

complex (diluted 50x; Vector Laboratories, Burlingame, CA, USA) for 3 h at RT, rinsed with PBS and incubated with 0.02% DAB in 50 mM Tris-HCl (pH 7.6) containing 0.002% H₂O₂ for 30 min at RT. The sections were then rinsed in distilled water and washed in 0.1 M PB. Sections were fixed with 1% osmium tetroxide (OsO₄) in 0.1 M PB for 40 min, rinsed in distilled water and counterstained with 1% (w/v) uranyl acetate for 30 min. Sections were then dehydrated through a graded series of ethanol 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).

Identification of the stages of spermatocyte

The spermatocyte is subdivided into 4 well-characterized phases termed 1) leptotene, 2) zygotene, 3) pachytene and 4) diplotene. In this study, we employed the pre-pachytene, pachytene and diplotene stages.

Western blot analysis

Spermatogenic cells were homogenized in 1 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM EDTA, 1% (w/v) SDS, 1 mM PMSF, 1% (w/v) sodium deoxycholate and 0.5% (w/v) protease inhibitor cocktail (Sigma Aldrich). Following centrifugation, the protein concentration was determined in the supernatants using the BCA protein assay (Pierce Chemical, Rockford, IL, USA). Aliquots containing 40 μ g total brain protein and 40 μ g or 60 μ g total testis protein were then boiled in loading buffer containing 50 mM Tris (pH 6.8), 6% 2-mercaptoethanol, 2% SDS, 10% glycerol and 0.004%

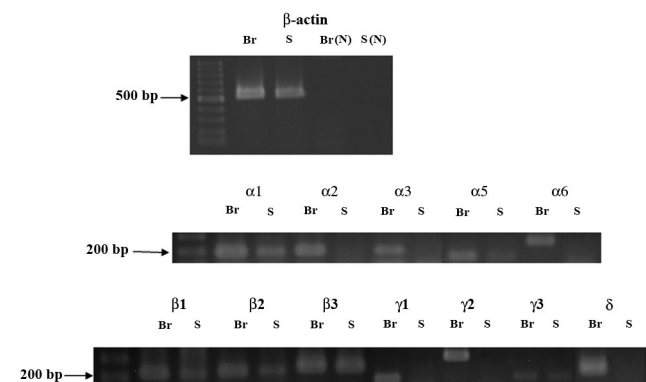


Fig. 1. RT-PCR analysis of the GABA_A receptor subunits α 1-6, β 1-3, γ 1-3, δ , and β -actin from mouse spermatogenic cells and brain tissue. S, spermatogenic cells; Br, brain. GABA_A subunits α 1, α 5, β 1-3, γ 3, and β -actin were expressed in the spermatogenic cells. The brain and testis samples were used without a reverse transcription step as negative control. N: Negative control.

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bromophenol blue. Each aliquot was then loaded onto an 8% polyacrylamide gel. After electrophoresis, the gels were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween-20 overnight, followed by incubation with goat anti-GABA_A receptor subunit α 1 IgG (diluted 100x,

Santa Cruz Biotechnology) or mouse anti-GAPDH (diluted 3200x, MAb 6C5, HyTest, Turku, Finland). Blocking peptide of GABA_A receptor α 1 subunit (diluted 100x, Santa Cruz Biotechnology) was used with anti-GABA_A receptor subunit α 1 IgG as negative control. After rinsing of the membranes, horseradish peroxidase-conjugated donkey anti-goat IgG or goat anti-mouse IgG (Santa Cruz Biotechnology) secondary

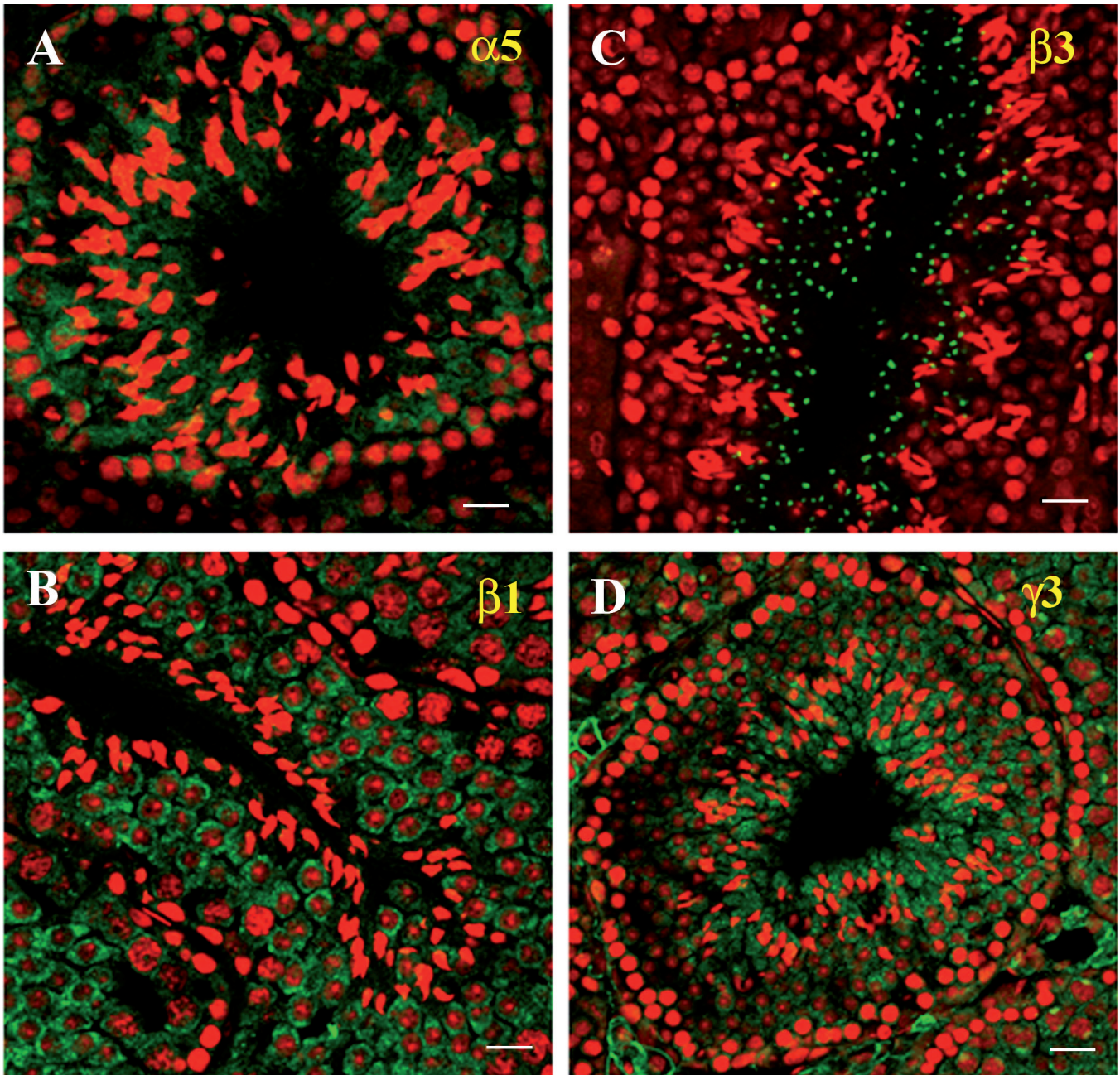


Fig. 2. Confocal microscopy of the GABA_A receptor subunits in murine seminiferous tubules (A-D). A, α 5; B, β 1; C, β 3; and D, γ 3. The α 5, β 1, β 3 and γ 3 represent stage VI-VII. The α 5, β 1 and γ 3 subunits were observed in the cytoplasm surrounding the nucleus in the spermatocytes and spermatids. The β 3 subunit was located in the residual cytoplasm of the spermatids during the maturation phase. Immunoreactivity is visible as green fluorescence and cell nuclei are stained with PI (red). Scale bar: 10 μ m.

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antibodies were applied and the chemiluminescent reaction carried out using ECL plus western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). The protein signals were detected using a LAS-3000 Lumio image analyzer (Fuji Photo Film, Tokyo, Japan). The signal intensities were further analyzed using multi gauge software (version 3.0; Fuji Photo Film).

Results

Analysis of GABA_A subunit mRNA in the mouse spermatogenic cells

We detected mRNA expression of β -actin and specific mRNA expression of the GABA_A subunits α 1, α 5, β 1-3 and γ 3 in the mouse spermatogenic cells using

RT-PCR (Fig. 1). The mRNA expression of β -actin was not detected in the brain and testis samples without a reverse transcription step. All GABA_A subunit mRNAs were detected in brain as positive control.

Confocal immunomicroscopy

Immunofluorescence confocal microscopy was used to investigate the spatial distribution of GABA_A subunits during spermatogenesis, from the spermatogonia to the elongated spermatid stages. Immunoreactivity for the α 5, β 1 and γ 3 subunits of GABA_A receptors was observed in the cytoplasm surrounding the nucleus in germ cells including the spermatocytes and spermatid. Immunoreactivity for the β 3 subunit was observed in the residual cytoplasm of the spermatids (Fig. 2). Immunoreactivity for the β 2 subunit was not detected in

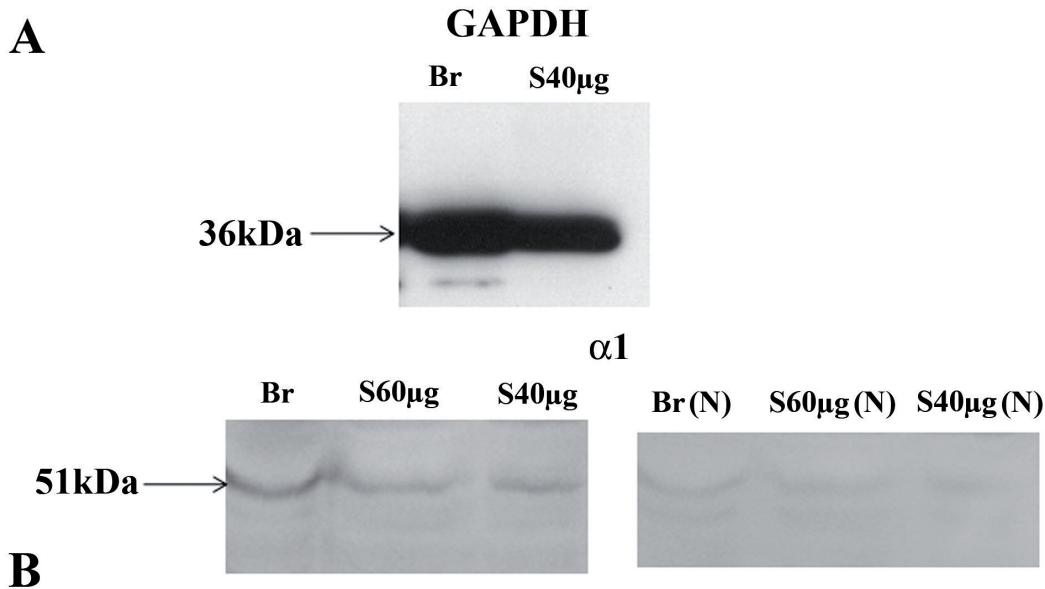
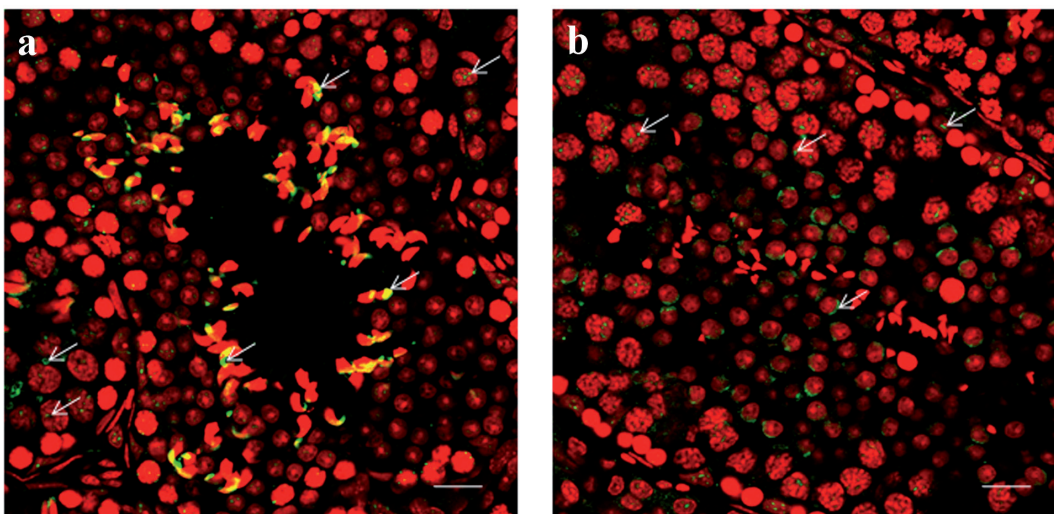


Fig. 3. Western blot analysis of the α 1 subunit (51 kDa) and GAPDH (36 kDa) in spermatogenic cells and brain (**A**) and confocal microscopy of the α 1 receptor subunit in murine seminiferous tubules (**B**). In part **A**, we identified a specific α 1 subunit protein band (51 kDa) in the mouse spermatogenic cells and brain. In part **B**, (a) represents stage II-III and (b) represents stage VI-VII. The presence of specific antibody bands for the α 1 subunit and GAPDH indicated by arrows were detected in the spermatogenic cells and brain samples. The α 1 subunit protein bands were absorbed by blocking peptide of the α 1 subunit antibody (negative control) (**A**). The α 1 subunit was expressed on the apical segment of the nucleus and in the nucleus (**B**). Immunoreactivity is visible as green fluorescence and the cell nuclei are stained with PI (red). White arrows indicate immunoreactivity. N: Negative control; S: Spermatogenic cells; Br: Brain. Scale bar: 10 μ m.



any of the germ cell populations (data not shown).

Western blot analysis and confocal immunomicroscopy of the α 1 subunit

Using western blot analysis, we identified a specific α 1 subunit protein band (51 kDa) and GAPDH (36 kDa) in the mouse spermatogenic cells and brain (positive control). The α 1 subunit protein bands were absorbed by blocking peptide of the α 1 subunit antibody (negative

control, Fig. 3A). We performed immunohistochemistry using a specific antibody directed against the α 1 subunit located in the mouse seminiferous tubules. α 1 subunit immunoreactivity was identified in the nuclei of the spermatogonia, spermatocytes and round spermatids, structures that were also stained with PI. In addition, staining was also located on the apical segment of the spermatids (Fig. 3B). Furthermore, spotted immunoreactivity was observed in the nuclei of the spermatogonia, pre-pachytene spermatocyte, pachytene

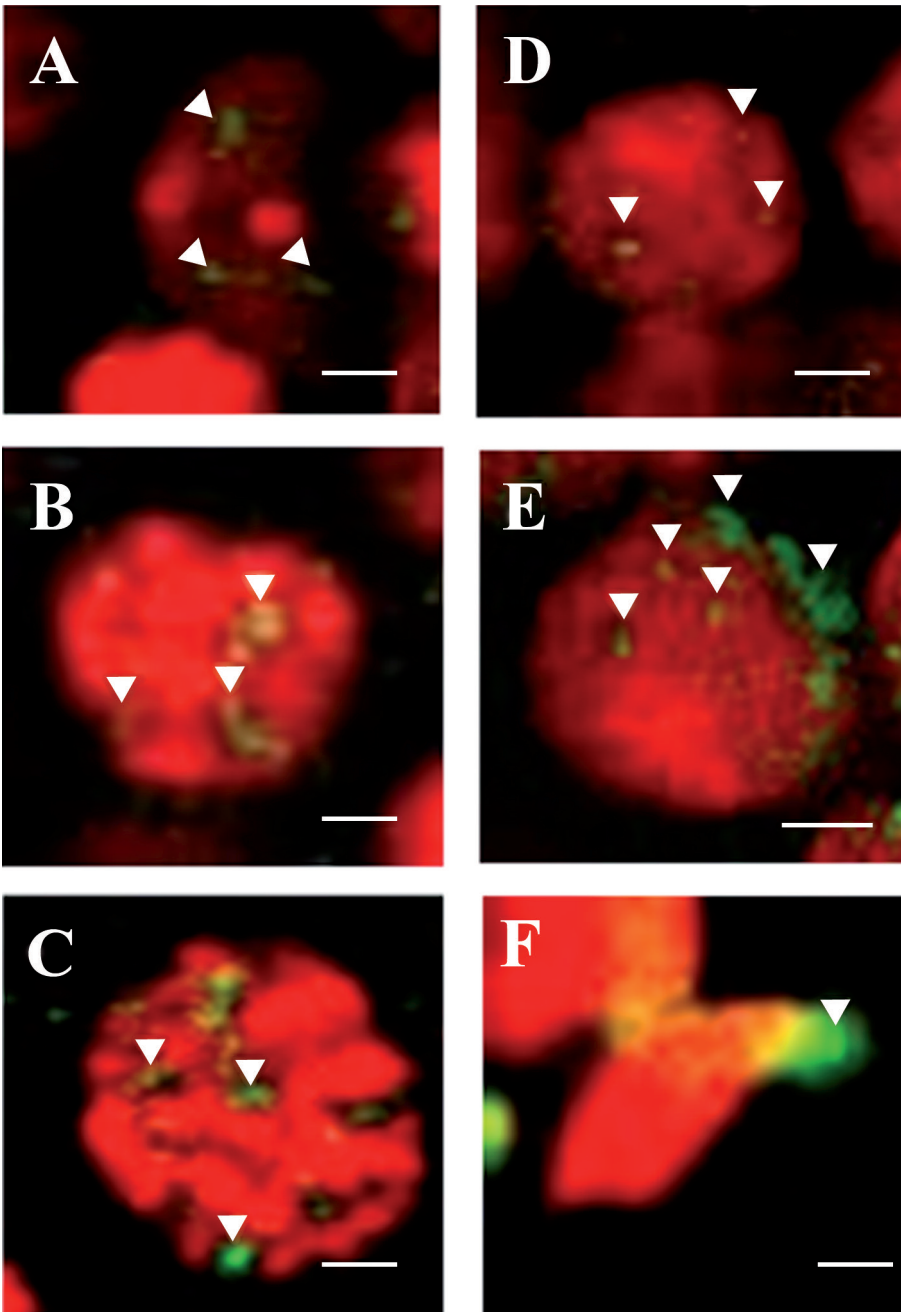


Fig. 4. Confocal images showing the cellular localization of α 1 subunit in each germ cell stage during spermatogenesis. **A**, spermatogonia; **B**, pre-pachytene spermatocyte; **C**, pachytene spermatocyte; **D and E**, round spermatid; and **F**, elongated spermatid. Immunoreactivity is visible as green fluorescence and the cell nuclei are stained with PI. Arrowheads show the punctate staining pattern observed in the nucleus of the spermatogonia (**A**) and spermatocytes (**B and C**). In the round spermatids (**D and E**), immunoreactivity was detected in the nucleus and acrosome. In elongated spermatids, immunoreactivity was detected only in the acrosome (**F**). Arrow heads indicate immunoreactivity. Scale bar: 1 μ m.

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spermatocyte and round spermatids (Fig. 4A-E). In the round and elongated spermatids, staining was identified in the acrosome (Fig. 4D-F).

Immunoelectron microscopy

The precise cellular distribution of the α 1 subunit during spermatogenesis was further investigated using immunoelectron microscopy. We found that the α 1 subunit was detected in the nucleus, but not the nucleolus, mitochondria or acrosome of the spermatocytes. In the spermatogonia, immunoreactive products were weakly stained in the nucleus (Fig. 5A). Furthermore, immunoreactive products were only very sparsely identified within the nucleus of the spermatocytes when compared to staining within the

spermatogonia (Fig. 5A-D). The intensity of the α 1 subunit staining appeared to be increased in the pachytene and diplotene spermatocytes when compared to that in the pre-pachytene spermatocyte (Fig. 5B-D). Within the nucleus of pachytene and diplotene spermatocytes, immunoreactive products were detected in the condensed chromatin rather than the extended chromatin, although the demarcation between the two areas was unclear, as the dense immunoreactive products were somewhat diffuse in nature. Immunoreactivity was also observed within the mitochondria (Fig. 5C,D). In the spermatids, α 1 immunoreactivity was detected in both the nucleus and acrosome structures (Fig. 6). In regards to the acrosome, immunoreactivity was observed in the acrosome vesicle and on the inner acrosomal membrane. The immunoreactivity observed in the

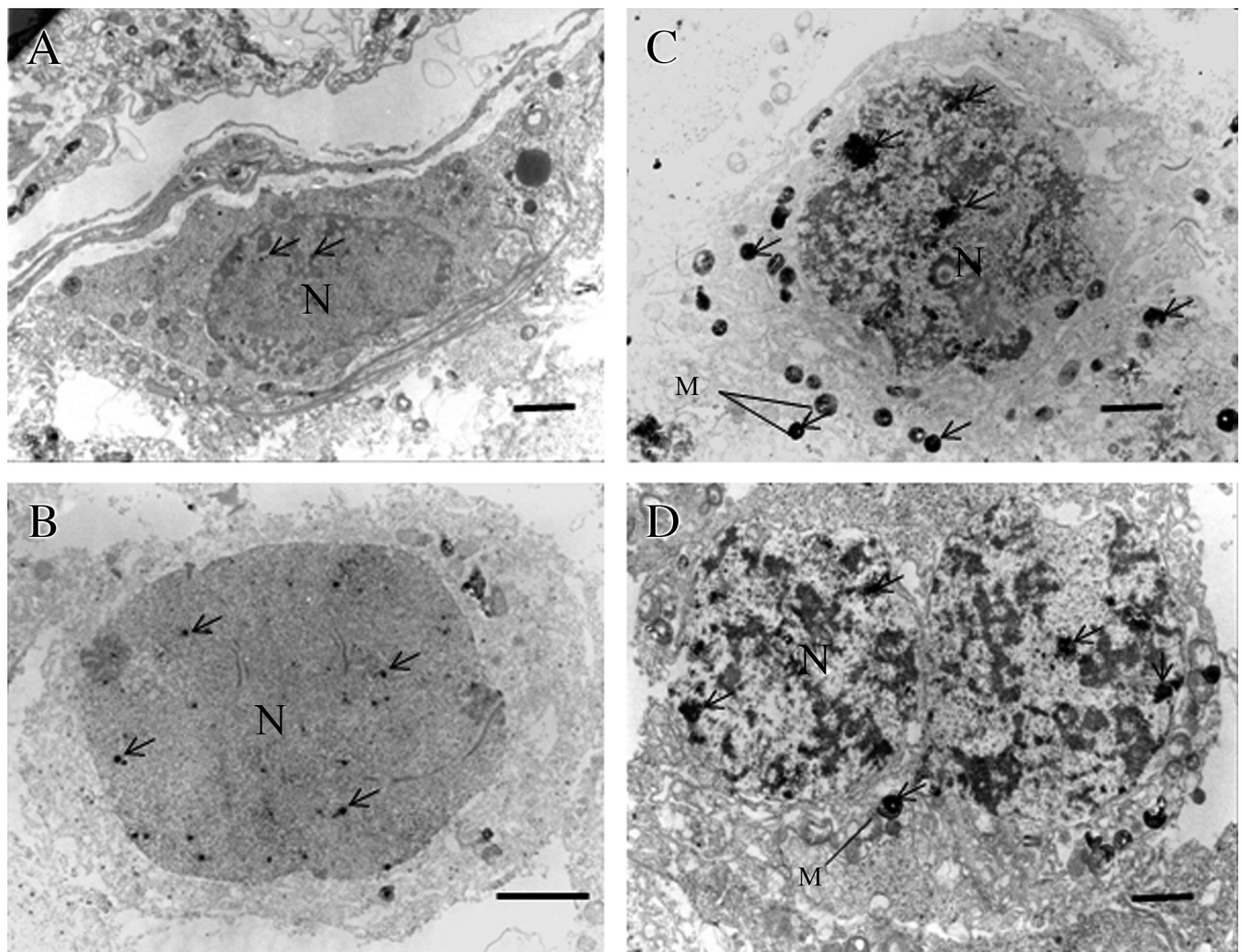


Fig. 5. Immunoelectron micrographs showing the distribution of the α 1 subunit in each germ cell stage during spermatogenesis. **A**, Spermatogonia; **B**, Pre-pachytene spermatocyte; **C**, Pachytene; and **D**, Diplotene spermatocyte. M: Mitochondria; N: Nucleus. Arrows indicate immunoreactivity. Scale bar: 1 μ m.

acrosome was maintained in the elongated spermatids (Fig. 6D). In contrast, the reactivity observed in the nucleus disappeared during nuclear elongation. The reactivity observed in the mitochondria was not identified at all in the spermatids.

Discussion

Until recently it was generally considered that the GABA_A receptors functioned merely as chloride channels that are pentamers in structure and comprised of several subunits, including α , β and γ subunits (McKernan and Whiting, 1996; Barnard et al., 1998; Costa, 1998; Mehta and Ticku, 1999). However, it is

now thought that the α 1 subunit of the GABA_A receptor alone may exhibit numerous important physiologic roles. In the NIH3T3 fibroblast cell line, expression of the α 1 subunit has been shown to be controlled by the transcriptional regulator c-myc. Up-regulation of the α 1 subunit induces a down-regulation in c-myc protein levels, which in turn increases cell proliferation. In contrast, over-expression of the α 1 subunit induces apoptosis in NIH3T3 cells (Vaknin and Hann, 2006). The distribution pattern of α 1 subunit in testicular germ cells appeared vastly different to that of the α 5, β 1 and γ 3 subunits. Staining of the α 5, β 1 and γ 3 subunits was observed in the cytoplasm surrounding the nuclei in the spermatocytes and spermatids during spermatogenesis,

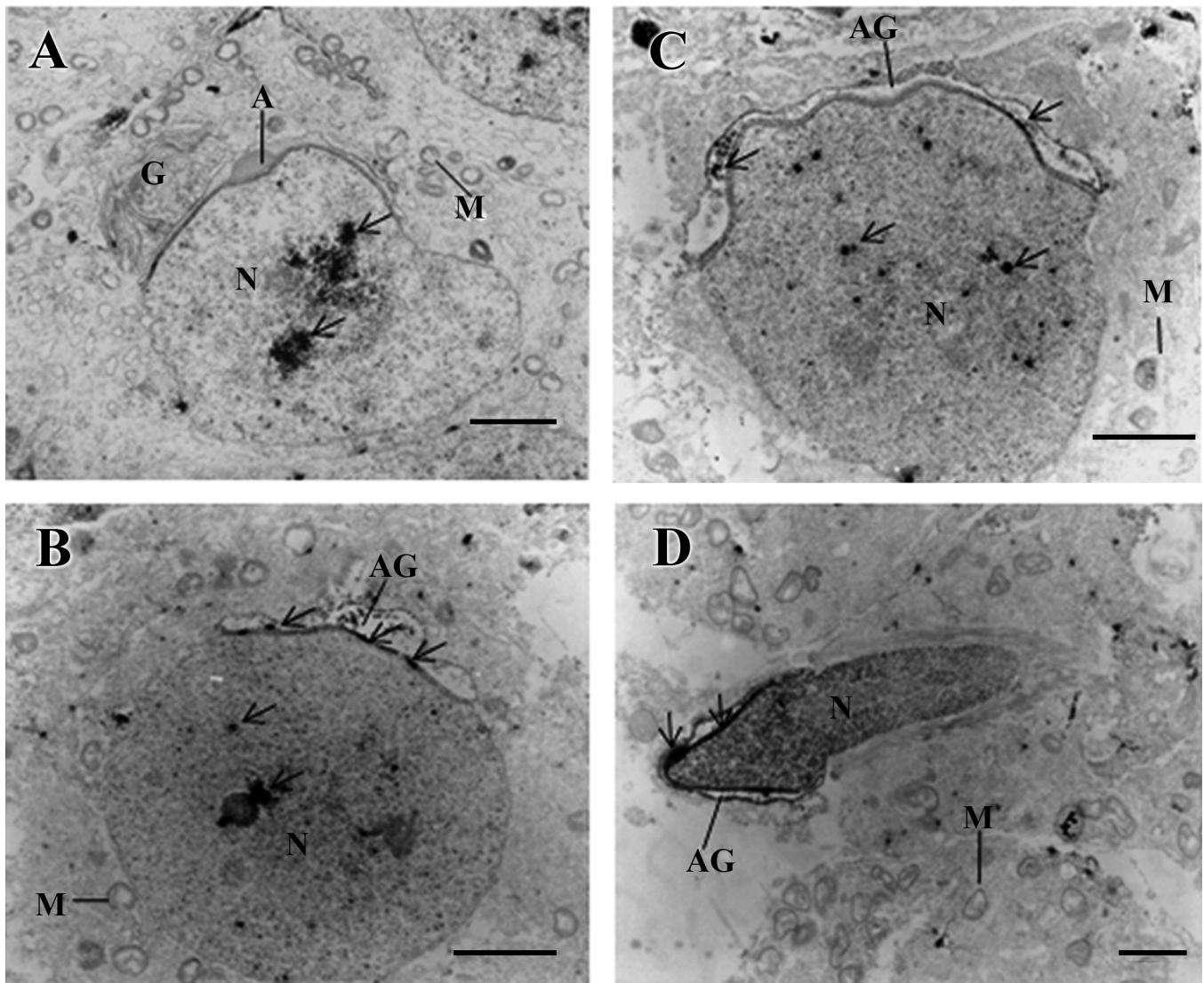


Fig. 6. Immunoelectron micrographs showing the distribution of the α 1 subunit in each spermatid phase during spermatogenesis. **A**, Golgi phase; **B**, Cap phase; **C**, Acrosome phase; and **D**, Maturation phase. In the late stage of spermatogenesis, the nucleus appears highly condensed. A: Acrosome; AG: Acrosomal granule; G: Golgi apparatus; M: Mitochondria; N: Nucleus. Arrows indicate immunoreactivity. Scale bar: 1 μ m.

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whereas substantial staining of the α 1 subunit was detected within the nucleus of the spermatogonia, spermatocytes and round spermatids. In particular, high levels of immunoreactivity of the α 1 subunit were observed in the nucleus of the pachytene spermatocyte. Furthermore, immunoelectron microscopy revealed that the α 1 subunit was mainly localized in the heterochromatin, the area of condensed chromatin, in the pachytene and diplotene spermatocytes.

Protein sequence analysis revealed that the α 1 subunit exhibits two specific amino acid sequences. One sequence encodes a nuclear localization signal, including KPKK, PEKPKKV and PKKVKDP. The second sequence encodes DM DNA binding domains (amino acid 160-209). DM DNA binding domains containing DM domain genes are present in both the human and mouse, and are also termed doublesex and mab-3 related transcription factors (Dmrts) (Kawamata and Nishimori, 2006; Kim et al., 2007; Murphy et al., 2007; Raymond et al., 2007). The *Dmrt* genes are thought to play important roles in sex determination events. *Dmrt1* and *Dmrt7* are known to be expressed in the testis, with *Dmrt1* required for testicular differentiation in adult mice (Kawamata and Nishimori, 2006; Raymond et al., 2007). The presence of DM DNA binding domains in the α 1 subunit raises the possibility that it may be involved in transcription processes that occur during maturation of the germ cells. This hypothesis is further supported by our data that demonstrate the protein levels of the α 1 subunit in the nucleus were highest in the pachytene and diplotene spermatocytes. It is well established that adult mammalian spermatogenesis is a highly coordinated process involving cellular proliferation, division, differentiation and programmed cell death. Programmed cell death or apoptosis occurs during the first meiotic division, which is one of the most strictly controlled steps of spermatogenesis.

During spermatogenesis, immunoreactivity of the α 1 subunit was also observed within the mitochondria of spermatocytes. Mitochondria play central roles not only in oxidative energy metabolism by producing the vast majority of cellular ATP, but also in apoptosis by orchestrating death signals triggered from both inside and outside of the cell (Honda and Hirose, 2003; Shaha, 2007). However, the precise physiological role of the GABA_A receptor α 1 subunit in the mitochondria remains largely unknown.

In the round and elongated spermatids, α 1 subunit immunoreactivity was detected in the acrosome. In the human male reproductive system, interaction between GABA and the GABA_A-like receptor has been shown to promote the acrosome reaction in spermatozoa (Calogero et al., 1996; de las Heras et al., 1997; Meizel 1997; Shi et al., 1997). The role of GABA_A-like receptors in the acrosome is therefore likely to be related to the acrosome reaction that occurs during fertilization. In addition, it is possible that these receptors may also play other important and independent roles during spermatogenesis.

In conclusion, our data suggest that the α 1 subunit plays an important modulator role during spermatogenesis. However, the precise physiological roles of the α 1 subunit remain unknown and thus further studies will be required to elucidate the molecular mechanisms underlying α 1 subunit activity during spermatogenesis.

References

- Abe H., Yanagawa Y., Kanbara K., Maemura K., Hayasaki H., Azuma H., Katsuoka Y., Yabumoto M. and Watanabe M. (2005). Epithelial localization of green fluorescent protein-positive cells in epididymis of the GAD67-GFP knock-in mouse. *J. Androl.* 26, 568-577.
- Akinci M.K. and Schofield P.R. (1999). Widespread expression of GABA_A receptor subunits in peripheral tissues. *Neurosci. Res.* 35, 145-153.
- Andäng M., Hjerling-Leffler J., Moliner A., Lundgren T.K., Castero-Branco G., Nanou E., Pozas E., Bryja V., Halliez S., Nishimaru H., Wilbertz J., Arenas E., Koltzenburg M., Charnay P., El Marira A., Ibanez C.F. and Ernfors P. (2008). Histone H2AX-dependent GABA_A receptor regulation of stem cell proliferation. *Nature Lett.* 451, 460-464.
- Barnard E.A., Skolnick P., Olsen R.W., Mohler H., Sieghart W., Biggio G., Braestrup C., Bateson A.N. and Langer S.Z. (1998). International union of pharmacology. XV. Subtypes of γ -aminobutyric acid_A receptors: Classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.* 50, 291-313.
- Calogero A.E., Hall J., Fishel S., Green S., Hunter A. and D'Agata R. (1996). Effects of gamma-aminobutyric acid on human sperm motility and hyperactivation. *Mol. Hum. Reprod.* 2, 733-738.
- Costa E. (1998). From GABA_A-receptor diversity emerges a unified vision of GABAergic inhibition. *Annu. Rev. Pharmacol. Toxicol.* 38, 321-350.
- de las Heras M.A., Valcarcel A. and Perez L.J. (1997). *In vitro* capacitating effect of gamma-aminobutyric acid in ram spermatozoa. *Biol. Reprod.* 56, 964-968.
- Fizman M.L. and Schousboe A. (2004). Role calcium and kinase on the neurotrophic effect induced by GABA. *J. Neurosci. Res.* 76, 435-441.
- Hayasaki H., Sohma Y., Kanbara K., Maemura K., Kubota T. and Watanabe M. (2006). A local GABAergic system within rat trigeminal ganglion cells. *Eur. J. Neurosci.* 23, 1-13.
- Honda S. and Hirose S. (2003). Stage-specific enhanced expression of mitochondrial fusion and fission factors during spermatogenesis in rat testis. *Biochem. Biophys. Res. Commun.* 311, 424-432.
- Kanbara K., Okamoto K., Nomura S., Kaneko T., Shigemoto R., Azuma H., Katsuoka Y. and Watanabe M. (2005). Cellular localization of GABA and GABA_B receptor subunit proteins during spermatogenesis in rat testis. *J. Androl.* 26, 485-493.
- Kawamata M. and Nishimori K. (2006). Mice deficient in *Dmrt7* show infertility with spermatogenic arrest at pachytene stage. *FEBS Lett.* 580, 6442-6446.
- Kim S., Bardwell V.J. and Zarkower D. (2007). Cell type-autonomous and non-autonomous requirements for *Dmrt1* in postnatal testis differentiation. *Dev. Biol.* 307, 314-327.
- Magnaghi V., Ballabio M., Cavarretta I.T.R., Froesti W., Lambert J.J., Zucchi I. and Melcangi R.C. (2004). GABA_B receptors in Schwann cells influence proliferation and myelin protein expression. *Eur. J. Neurosci.* 19, 2641-2649.

Cellular localization of GABA_A α 1

- McKerman R.M. and Whiting P.J. (1996). Which GABA_A-receptor subtypes really occur in the brain? *Trends Neurosci.* 19, 139-143.
- McCarrey J.R., Geyer C.B. and Yoshioka H. (2005). Epigenetic regulation of testis-specific gene expression. *Ann. NY Acad. Sci.* 1061, 226-242.
- Mehta A.K. and Ticku M.K. (1999). An update on GABA_A receptors. *Brain Res. Rev.* 29, 196-217.
- Meizel S. (1997). Amino Acid neurotransmitter receptor/ chloride channels of mammalian sperm and the acrosome reaction. *Biol. Reprod.* 56, 569-574.
- Murphy M.W., Zarkower D. and Bardwell V.J. (2007). Vertebrate DM domain proteins bind similar DNA sequences and can heterodimerize on DNA. *BMC Mol. Biol.* 8, 1-14.
- Raymond C.S., Murphy M.W., O'Sullivan M.G., Bardwell V.J. and Zarkower D. (2007). *Dmrt1*, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev.* 16, 2587-2595.
- Shaha C. (2007). Modulators of spermatogenic cell survival. *Soc. Reprod. Fertil. Suppl.* 63, 173-86.
- Shi Q.X., Yuan Y.Y. and Roldan E.R. (1997). γ -Aminobutyric acid (GABA) induces the acrosome reaction in human spermatozoa. *Mol. Hum. Reprod.* 3, 677-683.
- Tamayama T., Maemura K., Kanbara K., Hayasaki H., Yabumoto Y., Yuasa M. and Watanabe M. (2005). 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.* 273, 117-126.
- Tillakaratne N.J.K., Medina-Kauwe L. and Gibson K.M. (1995). Gamma-aminobutyric acid (GABA) metabolism in mammalian neural and nonneural tissues. *Comp. Biochem. Physiol.* 112A, 247-263.
- Vaknin U.A. and Hann S.R. (2006). The α 1 subunit of GABA_A receptor is repressed by c-myc and is pro-apoptotic. *J. Cell Biochem.* 97, 1094-1103.
- Wang J.M., Johnston P.B., Ball B.G. and Brinton R.D. (2005). The neurosteroid allopregnanolone promotes proliferation of rodent and human neural progenitor cells and regulates cell-cycle gene and protein expression. *J. Neurosci.* 25, 4706-4718.
- Watanabe M., Maemura K., Kanbara K., Tamayama T. and Hayasaki H. (2002). GABA and GABA receptors in the central nervous system and other organs. *Int. Rev. Cytol.* 213, 1-47.
- Xiouri M and Papazafiri P. (2006). Anti-apoptotic effects of allopregnanolone on P19 neurons. *Eur. J. Neurosci.* 23, 43-54.
- Zheng J., Xia X., Ding H., Yan A., Hu S., Gong X., Zong S., Zhang Y. and Sheng H.Z. (2008). Erasure of the paternal transcription program during spermiogenesis: The first in the reprogramming of sperm chromatin for zygotic development. *Dev. Dyn.* 237, 1463-1476.

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