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Expression of the $GABA_A$ receptor/Chloride Channel in murine spermatogenic cells

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Summary. Previous studies from our laboratory have demonstrated that γ-aminobutyric acid (GABA) and $GABA_B$ receptor subunits are expressed within the acrosome of spermatids during spermiogenesis. Furthermore, our previous study with the glutamate decarboxylase (GAD) 67-GFP knock-in mouse demonstrated that GFP–positive cells were localized to the epithelium of the caput of epididymis. In the present study, we detected $GABA_A$ subunits, including α1, α5, ß1-3 and γ3, and both isoforms of GAD, GAD65 and GAD67, in mouse spermatogenic cells using RT-PCR. The expression of these proteins was subsequently confirmed by western blot analysis. Immunohistochemistry also revealed that GABA, GAD65, and α5, ß1 and γ3 subunits of the $GABA_A$ receptor were localized in the membrane of spermatogenic cells, including spermatocytes and spermatids. The whole-cell patch-clamp analysis demonstrated that GABA application induced an inward chloride current in some of the large and round spermatogenic cells. Our findings show that spermatogenic cells have a GABA producing system by themselves, and that GABA may function via the ionotropic $GABA_A$ receptor. This data suggests that the GABAergic system may play important roles in the male reproductive system.

Key words: Spermatogenesis, $GABA_A$ receptor subunit, Patch-clamp, GABA

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Introduction

GABA is an inhibitory neurotransmitter in the central nervous system (CNS). GABA mediates its effects via two types of receptors, termed the ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors (Mott and Lewis, 1994; Rabow et al., 1995; Mehta and Ticku, 1999; Bowery and Enna, 2000; Calver et al., 2001; Watanabe et al., 2002). The $GABA_A$ receptor is a critical mediator of fast synaptic inhibition in the brain, and the predominant receptor subtype located in the CNS is thought to be a chloride channel composed of α, ß and γ subunits (McKerman and Whiting, 1996; Barnard et al., 1998; Costa, 1998; Mehta and Ticku, 1999). In neuronal tissues, activation of the $GABA_A$ receptor initiates signal transduction systems involving cAMP-dependent protein kinase (PKA), Ca^{2+}/phospholipid dependent protein kinase (PKC) (Poisbeau et al., 1999) and phospholipase C (PLC) (Frye and Walf, 2008).

In the female reproductive system, GABA has been shown to be present at high concentrations in the mammalian oviduct (Erdö and Wolff, 1990; László et al., 1992; Tillakaratne et al., 1995), while in the male reproductive system, interaction between GABA and $GABA_A$-like receptors has been shown to promote the hyperactivation and acrosome reaction of spermatozoa, an exocytotic event essential for fertilization (Lee and Storey, 1986; Shi and Roldan, 1995; Calogero et al., 1996; Shi et al., 1997; Ritta et al., 1998; Hu et al., 2002).

It is well established that intracellular Ca^{2+} concentration influences signal transduction pathways involving PKA, which in turn result in phosphorylation changes during the acrosome reaction (Breitbart, 2002). In addition, GABA receptor subunits and GAD 67

mRNAs have been detected by reverse transcription-polymerase chain reaction (RT–PCR) in testes and sperm during the early stages of spermatogenesis (Tillakaratne et al., 1992, 1995; Akinci and Schofield, 1999; Castelli et al., 1999; He et al., 2001; Hu et al., 2004). A recent report from our laboratory has demonstrated that GABA and $GABA_B$ receptor subunits are localized to the acrosome of spermatids during spermiogenesis (Kanbara et al., 2005). Furthermore, our study involving the GAD 67-GFP knock-in mouse showed that GFP-positive cells were expressed in the epithelium of the caput of epididymis (Abe et al., 2005). In combination, these reports suggest that the GABAergic system may play an important role in male reproductive system functions. The precise physiological role of the GABAergic system during spermiogenesis remains unknown. In the present study, we examined the expression and localization of GABA and $GABA_A$ receptor subunits during spermatogenesis using RT-PCR, western blot analysis and immunohistochemistry. In addition, to ascertain whether $GABA_A$ receptors in the spermatogenic cells are functional or not, we performed whole-cell patch-clamp experiments (Kirichok et al., 2006; Xiang et al., 2007).

Materials and methods

Animals

In this study, GAD67-GFP knock-in mice (Tamamaki et al., 2003) and BALB/c mice were used. The cDNA encoding enhanced GFP (EGFP) was inserted into exon 1 of the GAD67 gene by homologous recombination in GAD67-positive cells. EGFP was found to be expressed specifically in GAD67- positive neurons of a brain. The GAD67-GFP knock-in male mice and BALB/c male mice aged 7 to 8 weeks (Clea Japan, Osaka, Japan) were housed under standard conditions in a temperature controlled room and supplied with food (CE-2, Clea Japan) and water *ad libitum*. The experimental protocol was preapproved by the Ethics Review Committee for Animal Experimentation of Osaka Medical College.

Isolation of testicular cells

Mice were anesthetized with pentobarbital (50mg/kg, i.p; Abbott Laboratories, North Chicago, IL, USA). The testes were then dissected. In order to isolate the seminiferous tubules from the testes, 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. The seminiferous tubules were then harvested by low speed centrifugation of 800 rpm, washed three times in PBS without enzyme and cut into small fragments using two lancets. Tubular fragments were digested by the above solution including collagenase and DNase for 5 min, collected and then gently resuspended in PBS.

RNA isolation and RT-PCR

Total RNA was extracted from isolated testicular cells using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). cDNA was then synthesized using Omniscript Reverse Transcriptase (Qiagen GmbH) according to the manufacturer's instructions. The reverse transcription reaction mixture contained 1 μM oligo-d (T)12-18 primer, 10 units RNase inhibitor, 0.5 mM of each dNTP and 4 units Omniscript reverse transcriptase. PCR was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster, CA, USA). The PCR reaction mixture (25 μl) contained 1x GoTaq Green Master Mix reaction buffer (pH 8.5), 400 μM dNTP, 3 mM $MgCl_2$ (Promega, Madison, WI, USA), 2 μl cDNA solution and 0.2 μM of each primer. The primer sequences used for ß-actin, GAD65, GAD67 and $GABA_A$ α1-3, α5, α6, ß1-3, γ1-3 and δ subunits (Abe et al., 2005; Tamayama et al., 2005) are outlined in Table 1. The PCR amplification conditions consisted of 2 min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, 1 min at 72°C, and a final 5 min extension at 72°C. The PCR products were separated on 1.5% agarose gels then stained for 10 min with 0.1% ethidium bromide solution and illuminated using a UV transilluminator.

Immunohistochemical analysis

Mice were anesthetized with an intraperitoneal injection of pentobarbital, perfused transcardially with Ringer's solution and then fixed with 50 ml of 4% (w/v) paraformaldehyde and 0.05% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4). The testes were then dissected and immersed in the same fixative overnight at 4°C. Following brief rinsing with phosphate-buffered saline (PBS), the specimens were then immersed in 30% sucrose in PBS overnight at 4°C, embedded in OCT compound (Miles, Elkhart, IN, USA) and 7-μm sections were prepared by a cryostat microtome (Leica Microsystems, CM 3056, Germany, Nussloch).

We performed immunohistochemistry for $GABA_A$ receptor subunits, GAD and GABA using a goat polyclonal antibody directed against $GABA_A$ receptor subunits α1, α5, ß1-3 and γ3 (diluted 250x; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); rabbit polyclonal antibodies directed against GAD65 (diluted 1000x; Chemicon International, Temecula, CA, USA) and GABA (diluted 250x; Chemicon International); and a mouse monoclonal antibody directed against GAD67 (diluted 1000x; Chemicon International). The specificity of these antibodies has been reported previously (Hayasaki et al., 2006; Tamura et al., 2009). A rabbit monoclonal antibody directed against Cadherin (diluted 50x; Briefly, Molecular Probes, Eugene, OR, USA) was used as a marker of cell surface protein. We investigated the colocalization of $GABA_A$ receptor subunit α5, ß1 or γ3 with the cell surface marker by double-staining. Briefly, sections pre-washed with PBS were incubated

with normal donkey or goat serum (diluted 50x) for 30 min at room temperature (RT), and then incubated with each primary antibody overnight at 4°C. The sections were rinsed in PBS and incubated with Alexa Fluor™ 488 donkey anti-goat IgG secondary antibody (diluted 300x; Molecular Probes) for GABA$_A$ receptor subunits; Alexa Fluor™ 488 goat anti-rabbit IgG (diluted 300x; Molecular Probes) for GABA and GAD65; Alexa Fluor™ 488 goat anti-mouse IgG (diluted 300x; Molecular Probes) for GAD67; and Alexa Fluor® 546 goat anti-rabbit IgG (diluted 300x; Molecular Probes) for Cadherin for 60 min at RT in the dark. Sections were then rinsed with PBS. The sections, except for double-staining, were treated with 100 μg/ml RNase A in PBS for 1 h at 37°C and counterstained with 10 μg/ml of propidium iodide (PI, Molecular Probes) diluted in phosphoric and citric acid 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. Sections incubated with non-immune sera from the same species as the primary antibody served as a negative control.

Western blot analysis

The seminiferous tubules and whole brains were homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 5mM EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) sodium deoxycholate and 0.5% (w/v) protease inhibitor cocktail (Sigma Aldrich, Inc., St. Louis, MO63103, USA). Following centrifugation at 15000 rpm, supernatant protein concentration was determined using the BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). Aliquots containing 40 μg total brain protein, and 40 μg or 60 μg of seminiferous tubule protein were then boiled in loading buffer containing 50 mM Tris (pH 6.8), 6% 2-Mercaptoethanol, 2% SDS, 10% glycerol and 0.004% bromophenol blue. Each aliquot was then loaded onto an 8% polyacrylamide gel. Following electrophoresis, protein was transferred to PVDF membranes (Bio-Rad laboratories, CA, USA).

Once transferred, membranes were incubated in 5% skim milk diluted in PBS containing 0.1% Tween-20 overnight to reduce background staining. The filters were then incubated in goat anti-GABA$_A$ receptor subunit (α5, ß1, γ3) IgG diluted 1:100 (Santa Cruz Biotechnology) or mouse anti-GAPDH diluted 1:200 (MAb 6C5, HyTest, Turku, Finland) for 1h at RT. Blocking peptide for GABA$_A$ receptor α5, ß1 or γ3subunit (diluted 100X, Santa Cruz Biotechnology) was used with each anti-GABA$_A$ receptor subunit IgG as negative control. Following rinsing in PBS containing

Table 1. Primer sequences used for murine ß- actin, GAD65, GAD67 and GABA$_A$ receptor subunit RT-PCR.

Primers	Sequences	Product size	GenBank accession No.
GAD 65	F 5'-AGCCTTAGGGATTGGAACAG-3' R 5'-TTCCGGGACATCAGTAAC-3'	258	L16980
GAD 67	F 5'-GATACTTGGTGTGGCGTAGCCC-3' R 5'-ACGGGTGCAATTTCATATGTGAACATA-3'	575	Y12257
α1	F 5'-CAGCAAGAACTGTCTTTGGAG-3' R 5'-GCATACCCTCTCTTGGTGAA-3'	190	M86566
α2	F 5'-TCAGTGCTCGAAATTCCCT-3' R 5'-GTATCATGACGGAGCCTTTC-3'	195	M86567
α3	F 5'-ATGTCGTCATGACAACCCA-3' R 5'-GTTTCTGGCACTGATACTCAAG-3'	197	M86568
α5	F 5'-GTCTCCCTCTCAACAACCT-3' R 5'-GTAGAGAAGCGTGCCATCA-3'	140	AF540386
α6	F 5'-TGGGCAAACAGTTTCTAGTG-3' R 5'-AGCACTGATGCTTAAGGTG-3'	238	BC145702
ß1	F 5'-TGGCTATACCACGGATGAC-3' R 5'-AGTGTGGAAGGCATGTAGG-3'	222	BC130258
ß2	F 5'-GGCTACTTCATCCTGCAGA-3' R 5'-GAAGGGCCATAAAGACGAAG-3'	235	BC145975
ß3	F 5'-GCCATCGACATGTACCTGA-3' R 5'-GAATTCCTGGTGTCACCAAC-3'	260	BC153159
γ1	F 5'-CGGATGGGCTATTTCACGA-3' R 5'-CATTGCTGTCACGTAAGAAACC-3'	195	AF090375
γ2	F 5'-ACTTCCCAATGGATGAACAC-3' R 5'-TGTCATGGTCAGGACAGTAG-3'	375	M86572
γ3	F 5'-TCAAGCTGTCGAAAGCCA-3' R 5'-GTCTTTGCCATCCAGACACT-3'	219	BC138963
δ	F 5'-GCCAGAGTATCTCTAGGCA-3' R 5'-CGTTCCTCACATCCATCTC-3'	244	BC116836
ß- actin	F 5'-GTGGGCCGCTCTAGGCACCAA -3' R 5'-CTCTTTGATGTCACGCACGATTTC -3'	540	BC 138611

0.1% Tween-20, the blots were incubated in horseradish peroxidase-conjugated donkey anti-goat IgG or goat anti-mouse IgG (Santa Cruz Biotechnology) secondary antibodies and chemiluminescence was carried out using ECL plus western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). Protein signals were detected using a LAS-3000 lumio image analyzer (Fuji Photo Film, Tokyo, Japan), and the signal intensities were analyzed using Multi Gauge software (version 3.0, Fuji Photo Film).

Identification of isolated testicular cells from the seminiferous tubules

Cells obtained via dissociation of seminiferous tubules are easily recognized based on their cellular and nuclear morphology (Hagiwara and Kawa, 1984). The round spermatids are identified as small spermatogenic cells with pale nuclei. Pachytene spermatocytes of late primary spermatocytes are identified as the largest round spermatogenic cells with condensed nuclear chromatin. Pre-leptotene and leptotene spermatocytes of early primary spermatocytes are identified as intermediate size, round spermatogenic cells with slender thread chromatin. Interstitial cells are identified as large and polygonal cells, which are different from round spermatogenic cells.

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings for membrane ionic currents in identified spermatogenic cells were conducted using previously published methods (Hayasaki et al., 2006; Dave and Bordey, 2009). GABA-induced chloride channel currents were measured using a patch-clamp amplifier (EPC-8, HEKA elektronik, Lambrecht, Germany) which was controlled by a Macintosh computer (Power Macintosh G3, Apple Computer, Cupertino, CA, USA) equipped with an interface (ITC-16, Instrutech, Elmont, NY, USA) and control software (Pulse+PulseFit, HEKA elektronik). Data was stored on a DAT recorder (Model 5870, NF, Tokyo, Japan). Patch pipettes were pulled from borosilicate glass capillary tubes (GC120F15, Clark Electromedical Instruments, Pangbourne, UK) using a vertical electrode puller (PP-83, Narishige Scientific Instrument Laboratories, Tokyo, Japan) and exhibited a resistance between 4 and 6 MΩ. The filling solution of the patch-pipettes contained 150 mM CsCl, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM EGTA (pH 7.4 adjusted with CsOH). The bath solution consisted of 145 mM NaCl, 4.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, and 20 mM Sucrose (pH 7.4 adjusted with 1 N HCl). The membrane potential was maintained at -60 mV during the recording. The current records were played back and low-pass filtered (902LPF, Frequency Devices, Haverhill, USA) at 300 Hz and digitized at 1,000 samples/s with PowerLab 2/25 (ADInstruments, Castle Hill, New South Wales, Australia). The data obtained was analyzed using the AXOGRAPH program system (Axon Instruments, Foster City, CA, USA). Currents flowing from the extracellular to the cytoplasmic side were defined as inward, and shown as upward deflection in figures. The concentrations of GABA applications were from 10 μM to 300 μM. For inhibitor study, bicuculline of $GABA_A$

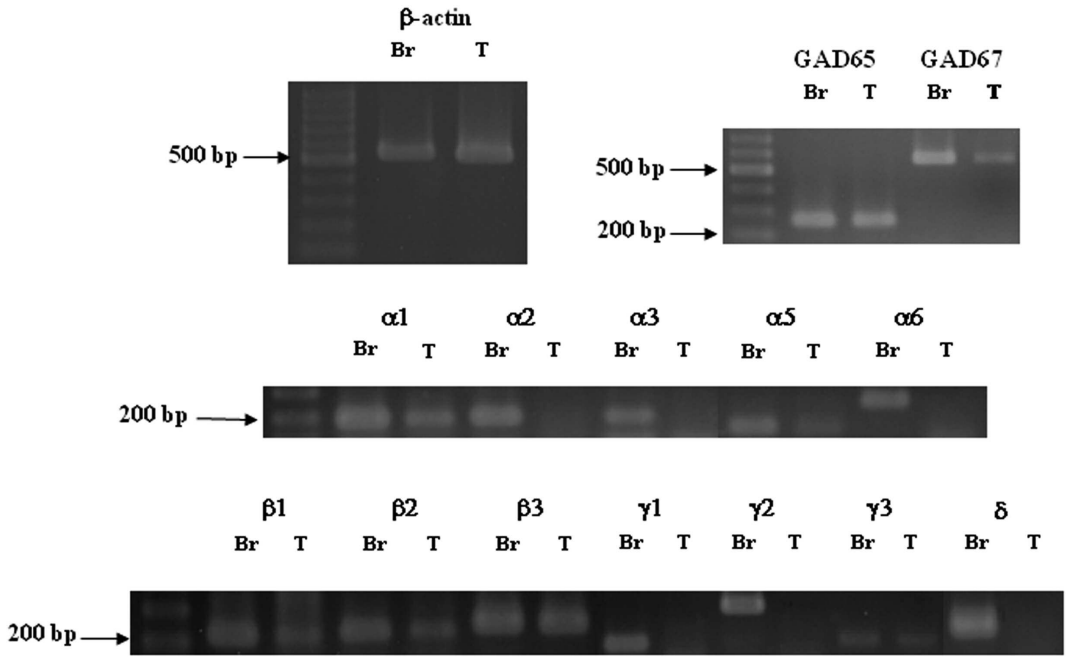

Fig. 1. RT-PCR analysis of ß-actin, GAD65, GAD67 and $GABA_A$ receptor subunit mRNA (α1-6, ß1-3, γ1-3 and δ subunits) from mouse testicular cells and brain tissue. ß-actin, $GABA_A$ subunits α1, α5, ß1-3 and γ3, and both GAD65 and GAD67 isoforms of GAD were expressed in the testicular cells. Br, brain; T, testicular cells.

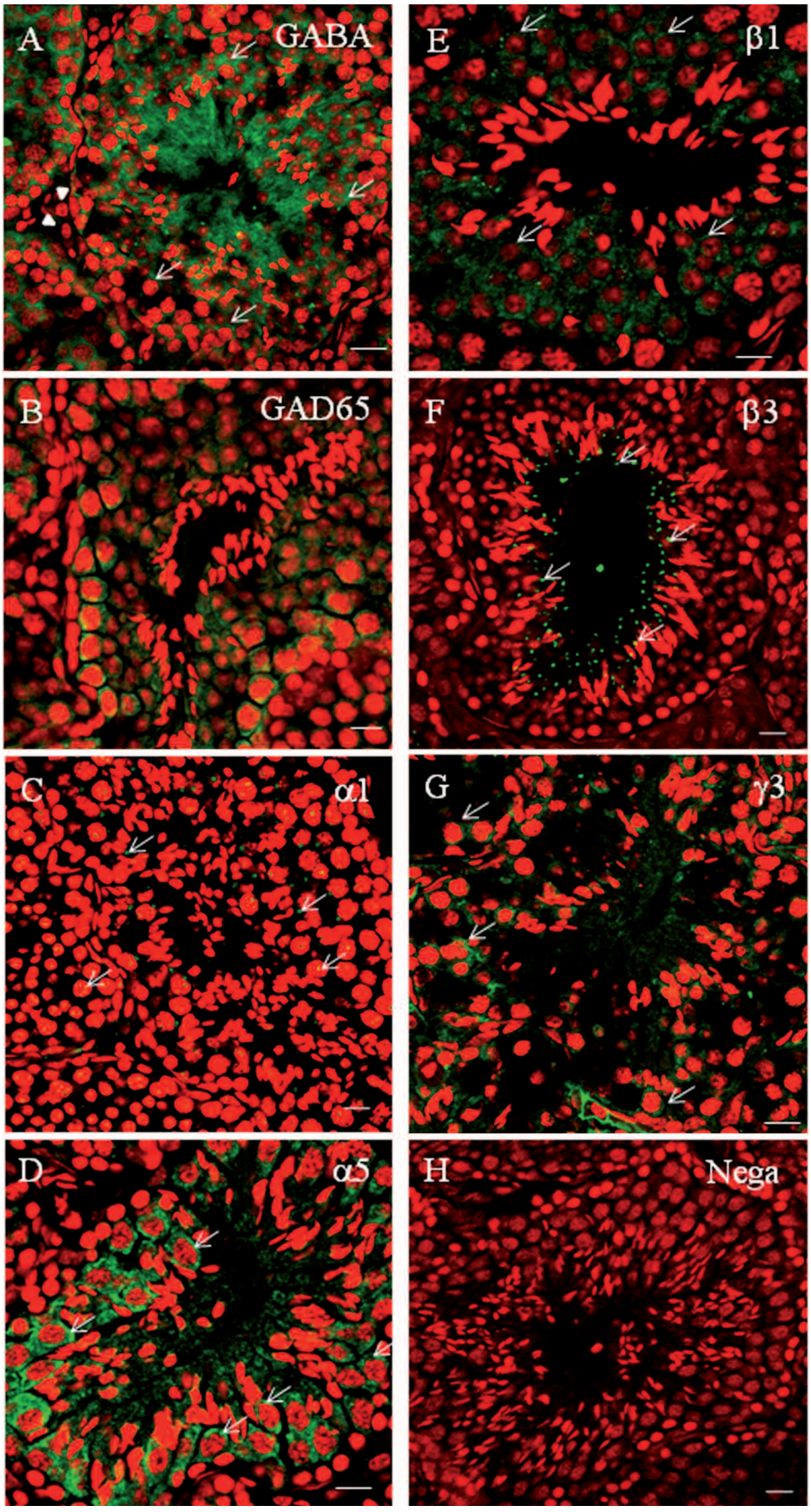

Fig. 2. Confocal laser microscopy images of GABA, GAD and $GABA_A$ receptor subunit expression in the mouse seminiferous tubules (**A - G**). **A.** GABA. **B.** GAD65. **C.** α1. **D.** α5. **E.** ß1. **F.** ß3. **G.** γ3. GABA, GAD65 and the α5, ß1 and γ3 subunits were expressed in the cytoplasm surrounding the nucleus or the membrane in the spermatocytes and spermatids. The α1 subunit was expressed on the apical segment of the nucleus and in the nucleus. The ß3 subunit was located in the residual cytoplasm of the spermatids during the maturation phase. GABA was not detected in interstitial cells (**A**). The non-immune sera which served as a negative control (Nega) showed no specific staining (**H**). Immunoreactivity was observed as a green color reaction. Arrows indicate immunoreactivity. Arrow heads indicate interstitial cells. Nuclei of all cells were counterstained with PI (red in color). Scale bar: 10 μm.

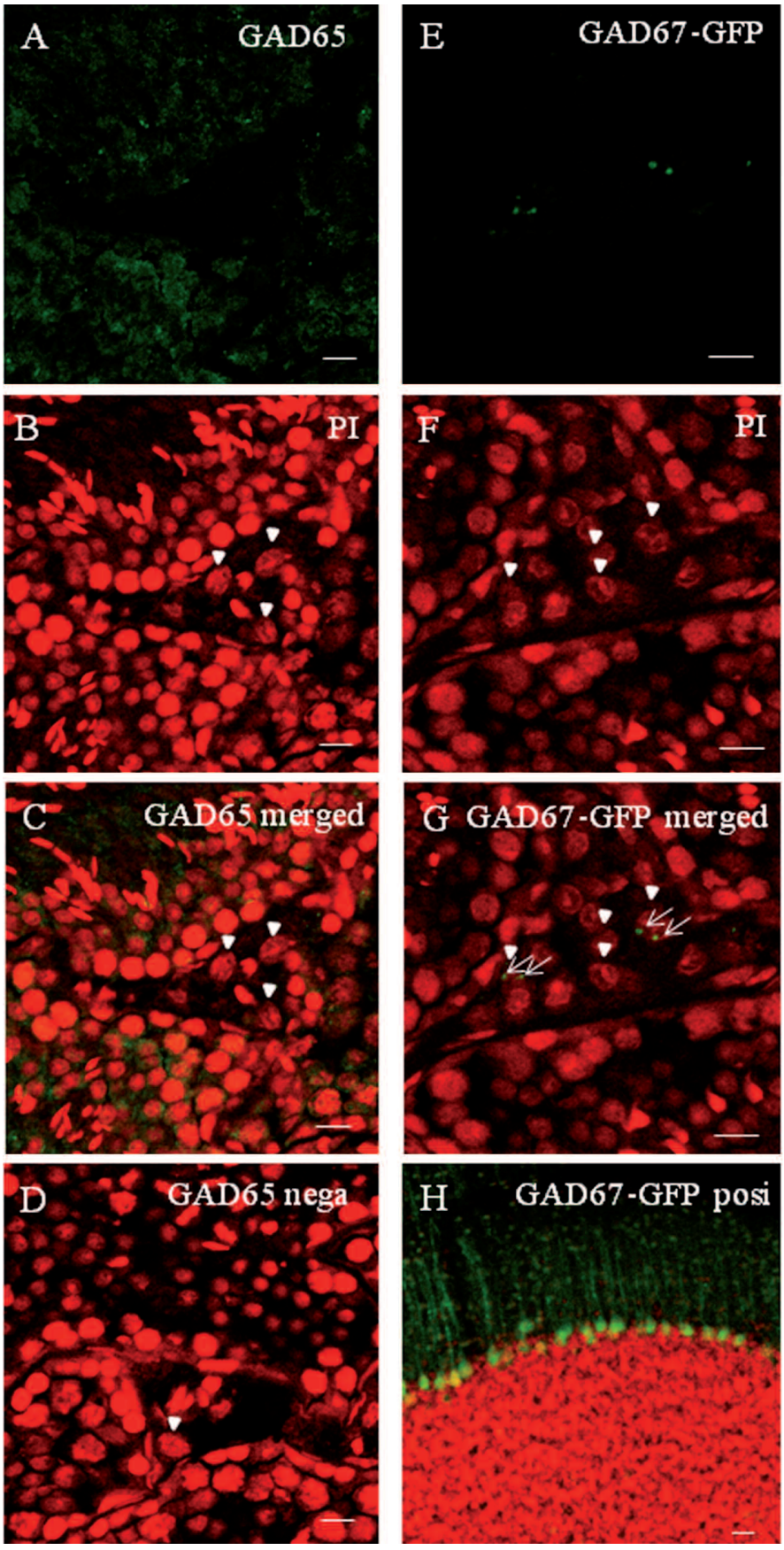

Fig. 3. Immunohistochemical staining for GAD 65 (**A - C**) and GAD67-GFP fluorescence (**E - G**) in interstitial cells of testis. No immunoreactivity for GAD65 was detected in interstitial cells **(A and C)**. The non-immune sera which served as a negative control (Nega) showed no specific staining (**D**). Faint and punctate GFP-fluorescence was observed in some interstitial cells of GAD67-GFP knock-in mice testis (**E and G**). GAD67- positive neurons in the cerebellum are shown as positive control (**H**). Immunoreactivitties for GAD65 and GAD67- positive cells were observed as a green color reaction. Arrows indicate GAD67 reaction in interstitial cells. Arrow heads indicate interstitial cells. Nuclei of all cells were counterstained with PI (red in color). Scale bar: 10 μm.

antagonist was used.

Results

PCR analysis

We detected mRNA expression of ß-actin, $GABA_A$ subunits α1, α5, ß1-3 and γ3, and both GAD65 and GAD67 isoforms of GAD in the testicular cells using RT-PCR. ß-actin, GAD and $GABA_A$ subunit mRNA expression was also detected in the brain tissue, the expression of which served as a positive control (Fig. 1).

Immunohistochemical and GAD 67-GFP fluorescent analyses

We examined the distribution of $GABA_A$ receptor subunits and GADs detected by RT-PCR, and GABA (Fig. 2). Immunoreactivities for GABA, GAD65 and $GABA_A$ receptor α5, ß1 and γ3 subunits were found in the cytoplasm surrounding the area of the nucleus or the membrane of male germ cells, including the spermatocytes and spermatids. In contrast, immunoreactivity for the α1 subunit was observed on the apical segment of the nucleus and in the nucleus of the spermatocytes and spermatids. Immunoreactivity for the ß3 subunit was confined to the residual cytoplasm of the spermatids, taking on a spot-like appearance during the maturation phase. Immunoreactivities for the ß2 subunit and GAD67 were not detected in any of the germ cell populations observed (data not shown). Immunoreactivities for GABA and GAD65 were not detected in interstitial cells (Figs 2A, 3A-C). Faint and punctate GFP-fluorescence was observed in some interstitial cells of GAD67-GFP knock-in mice testis (Fig. 3E-G). GAD67- positive neurons in the cerebellum were shown as positive control (Fig. 3H). The non-immune sera which served as a negative control showed no specific staining (Fig. 2H and Fig. 3D).

Western blot analysis

The specific antibody bands for the α5 subunit (54 kDa), ß1 subunit (57 kDa) and γ3 subunit (50 kDa) were detected in the seminiferous tubules and brains (positive control) (Fig. 4). The α5, ß1 or γ3 subunit protein bands

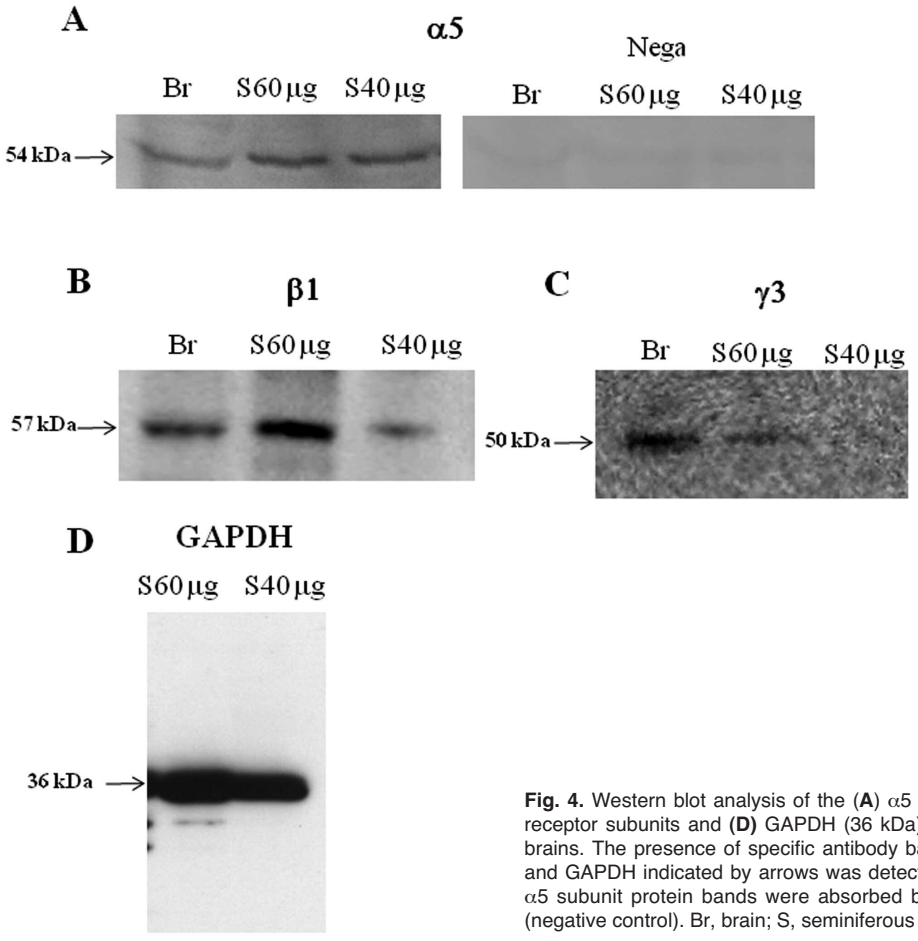

Fig. 4. Western blot analysis of the (**A**) α5 (54 kDa), (**B**) ß1 (57 kDa) and (**C**) γ3 (50 kDa) receptor subunits and **(D)** GAPDH (36 kDa) in the mouse seminiferous tubules and whole brains. The presence of specific antibody bands for the α5 subunit, ß1 subunit, γ3 subunit and GAPDH indicated by arrows was detected in the seminiferous tubules and brains. The α5 subunit protein bands were absorbed by blocking peptide of the α5 subunit antibody (negative control). Br, brain; S, seminiferous tubule; Nega, negative control.

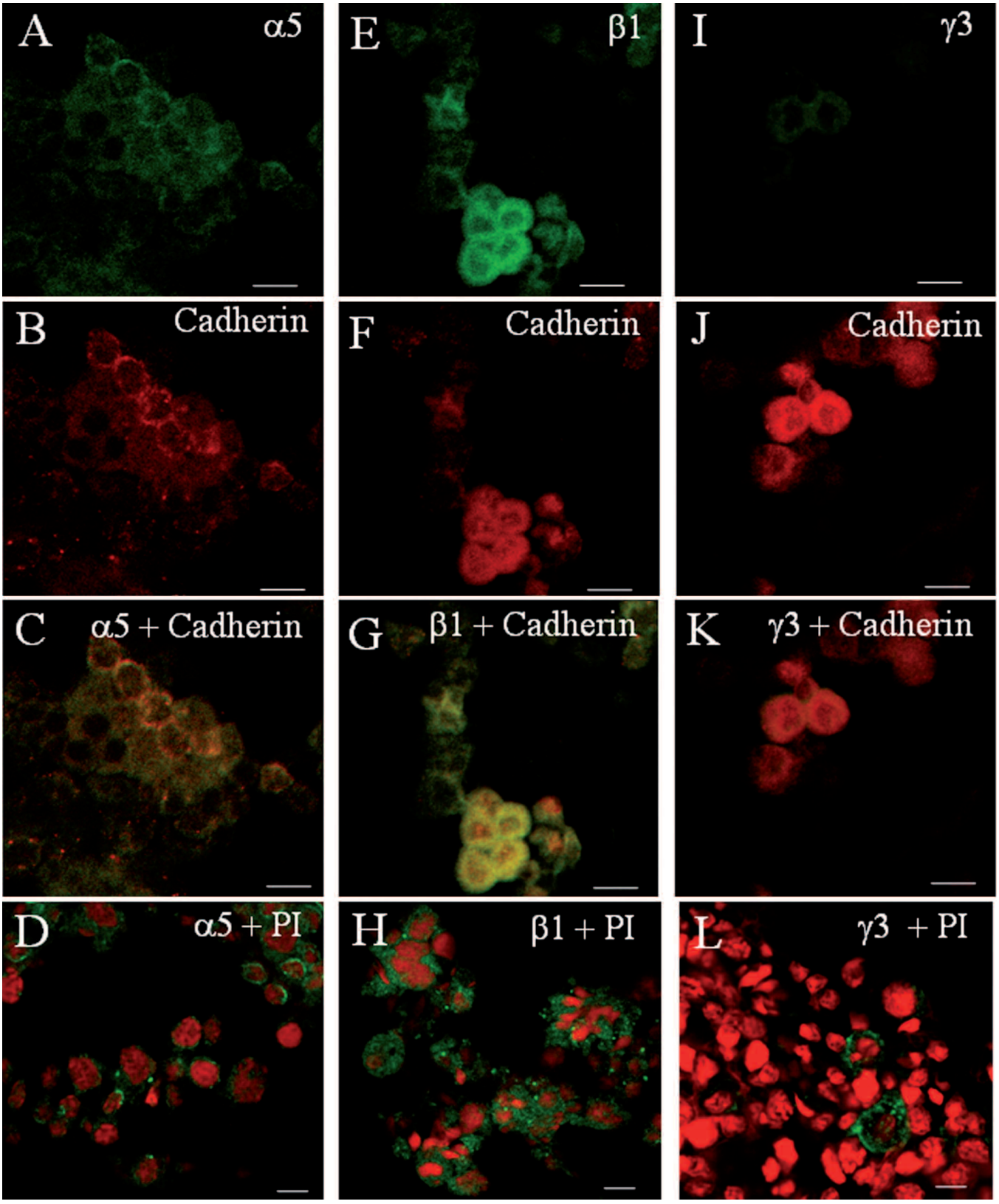

Fig. 5. Confocal laser microscopy images of the α5, ß1 and γ3 receptor subunits, and Cadherin as a marker of cell surface protein in spermatogenic cells isolated from the seminiferous tubules. **A-D.** α5. **E-H.** ß1. **I-L.** γ3. **B, F and J.** Cadherin. **C, G and K.** Merged images of each receptor subunit and Cadherin. The cell membrane stained with Cadherin is shown in red. The immunoreactivities of the α5, ß1 and γ3 receptor subunit were visible as green fluorescence and the cell nuclei stained with PI is shown in red (**D, H and L**). The α5, ß1 and γ3 receptor subunits were expressed in spermatogenic cells. However, the distribution of γ3 subunit immunoreactivity demonstrated a heterologous pattern in the spermatogenic cells in comparison with α5 and ß1 subunits (**D, H and L)**. In the double-staining, $GABA_A$ subunit-positive cells were also positive for Cadherin (**C, G and K**). Scale bar: 10 μm.

were absorbed by blocking peptide of each subunit antibody (negative control, Fig. 4A). The data of negative controls for ß1 and γ3 was not shown.

Primary culture

Spermatogenic cells obtained from seminiferous tubules showed substantial immunoreactivities of α5, ß1 and γ3 subunits (Fig. 5). However, the distribution pattern of the γ3 subunit was heterologous in the separated spermatogenic cells in comparison with the distribution patterns of α5 and ß1 subunits (Fig. 5D,H and L). In the double-staining, GABA$_A$ subunit-positive cells were also positive for Cadherin, which was used as a marker of cell surface protein (Fig. 5C,G,K).

Electrophysiological findings

To determine whether GABA$_A$ receptors function as chloride ion channels in the spermatogenic cells isolated from the seminiferous tubules, we performed whole-cell patch-clamp experiments (Fig. 6). The currents for low concentrations of GABA applications were not detected (data not shown). As is shown in Fig. 6A, applications of GABA (300 μM) to the bath induced inward currents at a holding potential of -60 mV. The GABA-evoked inward current was reproducible in the large and round spermatogenic cells. After washing, the current disappeared. Only a few cells (5 of the 15) responded to GABA application. These currents were not observed by bicuculline application, which was an antagonist for GABA$_A$ receptors (Fig. 6B).

Discussion

GABA$_A$ receptors in the CNS are mainly comprised of α, ß or γ subunits, and function as chloride channels (McKerman and Whiting, 1996; Barnard et al., 1998; Costa, 1998; Mehta and Ticku, 1999). In the male reproductive system, interaction between GABA and

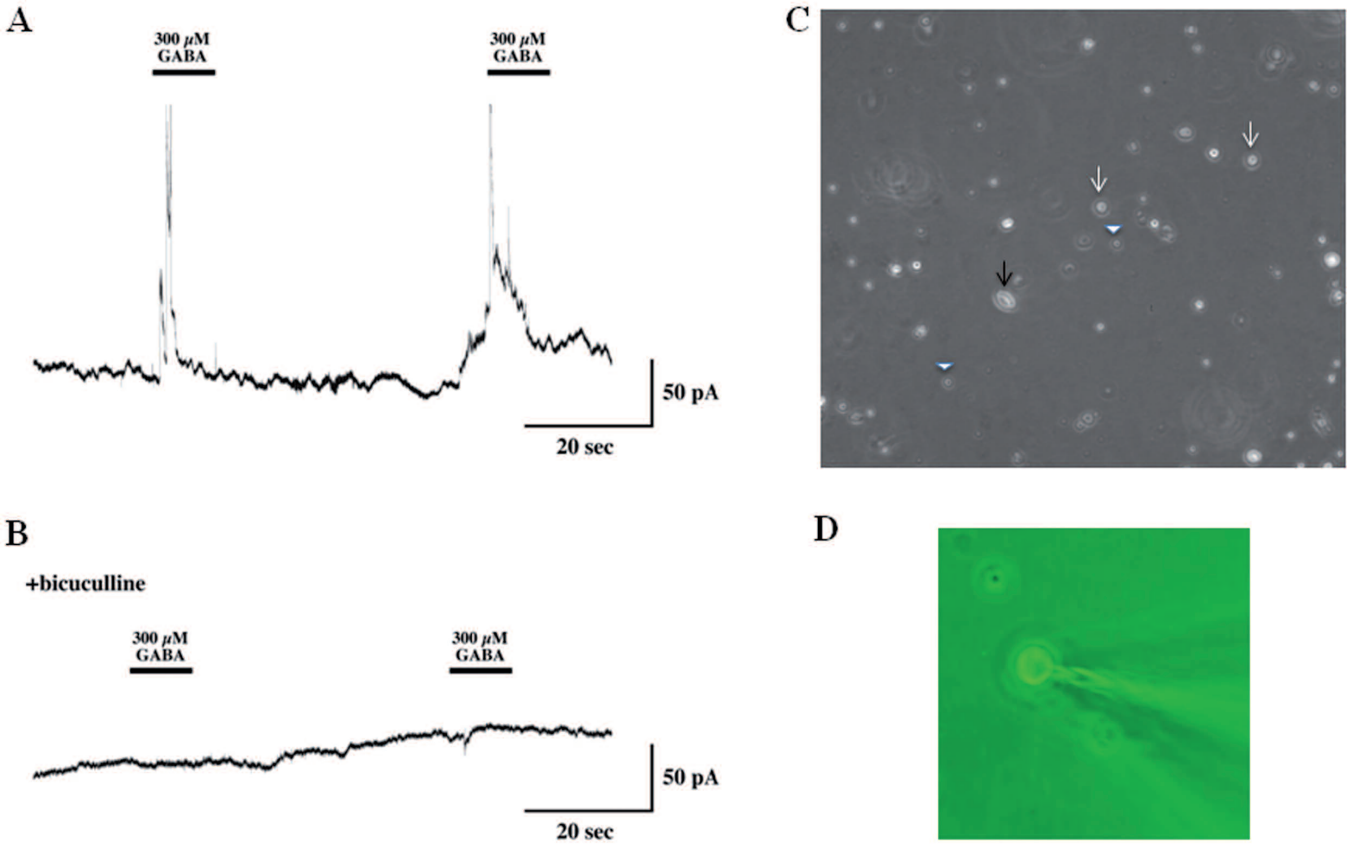

Fig. 6. GABA-induced chloride channel currents generated by whole-cell patch-clamp analysis. Vm = -60mV. GABA concentration: 300 μM. GABA-evoked inward currents were detected in 5 of the 15 isolated large and round spermatogenic cells (**A**). Currents by the bicuculline application, an antagonist for GABA$_A$ receptors (**B**). Bicuculline concentration: 10 μM. GABA-induced chloride channel currents were not observed by bicuculline application. Identification of isolated testicular cells (**C**). Pachytene spermatocytes indicated by white arrows were identified as the largest spermatogenic cells with condensed nuclear chromatin. White arrowheads indicate round spermatids. Black arrow indicates interstitial cell. Whole-cell patch-clamp photograph of pachytene spermatocyte (**D**).

$GABA_A$-like receptors has been shown to promote the hyperactivation and acrosome reaction in spermatozoa, an exocytotic event that is essential for fertilization (Lee and Storey, 1986; Shi and Roldan, 1995; Calogero et al., 1996; Shi et al., 1997; Ritta et al., 1998; Hu et al., 2002). In the present study, we demonstrated that $GABA_A$ receptor subunits and GAD, an enzyme which mediates GABA synthesis, were expressed in mouse testicular cells by RT-PCR (Fig. 1). Further immunohistochemical analysis also revealed the presence of GABA in spermatogenic cells and demonstrated that the α5, ß1 or γ3 subunits were localized in the whole of the membrane of spermatogenic cells, including spermatocytes and spermatids, by double staining with cell surface marker (Figs. 2, 5). Western blot analysis clarified the presence of these subunit proteins in the mouse seminiferous tubules (Fig. 4). This data indicates that spermatogenic cells possess a functional GABAergic system. In contrast, immunoreactivities for GABA and GAD65 were not detected in interstitial cells, including Leydig cells (Figs. 2A, 3A-C). Faint and punctate GFP-fluorescence was observed in some interstitial cells of GAD67-GFP knock-in mice (Fig. 3 E-G). This was a little different from some reports that GABAergic system was present in Leydig cells (Geigerseder et al., 2004; Doepner et al., 2005; Liu et al., 2009). The role of the GABA system in the interstitial tissue of mice remains unknown in the present study.

The whole-cell patch-clamp experiments undertaken in the present study revealed that GABA application was able to induce an inward chloride current in large and round spermatogenic cells (Fig. 6). These findings were in accordance with those also obtained by Xiang et al. (2007) and indicate that chloride channels that consist of $GABA_A$ receptor subunits are able to open in the presence of GABA. However, the chloride channel current response was low following GABA application to primary cultured spermatogenic cells in the present study. The α, ß and γ subunits are needed so that $GABA_A$ receptors function as a chloride channel. Immunohistochemical analysis of these cells revealed that primary cultured spermatogenic cells seem to demonstrate heterologous expression of the γ3 subunit, perhaps contributing to this result (Fig. 5I -L).

The precise function of the GABAergic system, comprising the GABA production system and $GABA_A$ receptors in spermatogenic cells remains unclear. It has been demonstrated that GABA promotes synaptogenesis in the cervical ganglion of the rat and may influence neuronal differentiation (Wolff et al., 1978). In cultured embryonic hippocampal and neocortical neurons, $GABA_A$ receptor activation has been shown to promote neurite outgrowth and maturation of GABA interneurons through membrane depolarization and increases in calcium level (Barbin et al., 1993; Marty et al., 1996; Maric et al., 2001; Dave and Bordey, 2009). Activation of the $GABA_A$ receptor involves the signal transduction system, including PKA, PKC (Poisbeau et al., 1999) and PLC (Frye and Walf, 2008). Thus, in neuronal tissues, the GABAergic system comprising the $GABA_A$ receptor is thought to be involved in cell proliferation, migration and differentiation via GABA-mediated signaling.

Similar functions have also been suggested in some peripheral non-neuronal tissues, such as chondrocytes, synovial membrane, and epithelial cells of the rat jejunum (Wang et al., 2004; Tamayama et al., 2005; Tamura et al., 2009). In the male reproductive system, $GABA_A$ receptor-like/chloride channels and GABA have also been shown to play essential roles in the acrosome reaction during fertilization (Meizel, 1997). In combination with these findings, our report strongly suggests that interaction between GABA and $GABA_A$ receptor may also play an important functional role in the male reproductive system (Darszon et al., 1999). Moreover, it has been proposed that the GABAergic system containing the $GABA_A$ receptors may be involved in proliferation, migration and differentiation events, as well as the promotion of cell survival via the appropriate signaling pathways during spermatogenesis (Xiouri and Papazafiri, 2006; Watanabe et al., 2006; Xiang et al., 2007). However, the precise roles of GABA signaling during spermatogenesis remain unknown. Future studies involving $GABA_A$ receptor deficient mutant mice or germ cell lines may help to clarify the precise mechanisms underlying GABA signaling effects on spermiogenesis.

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