

Dynamic regulation of glutamic acid decarboxylase 65 gene expression in rat testis

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Glutamate decarboxylase 65 (GAD65) produces γ -aminobutyric acid, the main inhibitory neurotransmitter in adult mammalian brain. Previous experiments, performed in brain, showed that *GAD65* gene possesses two TATA-less promoters, although the significance is unknown. Here, by rapid amplification of cDNA ends method, two distinct *GAD65* mRNA isoforms transcribed from two independent clusters of transcription start sites were identified in post-natal rat testis. RT-PCR results revealed that the two mRNA isoforms had distinct expression patterns during post-natal testis maturation, suggesting that *GAD65* gene expression was regulated by alternative promoters at the transcription level. By using GAD65-specific antibodies, western blotting analysis showed that the 58-kDa GAD65, N-terminal 69 amino acids truncated form of full-length GAD65 protein, was developmentally expressed during post-natal testis maturation, suggesting that *GAD65* gene expression in testis may also be regulated by post-translational processing. Confocal immunofluorescence microscopy revealed that GAD65 protein was presented in Leydig cells of Day 1 testis, primary spermatocytes and spermatids of post-natal of Day 90 testis. The above results suggested that *GAD65* gene expression is dynamically regulated at multiple levels during post-natal testis maturation.

Keywords transcription; gene regulation; glutamate decarboxylase; testis

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Introduction

Glutamate decarboxylase (GAD) is the key enzyme in the synthesis of the inhibitory neurotransmitter

γ -aminobutyric acid (GABA). Encoded by separate genes, two isoforms of GAD, namely GAD65 (65 kDa) and GAD67 (67 kDa), are found in mammalian central nervous system (CNS). GAD has also been demonstrated in a variety of peripheral tissues, including pancreas and testis [1,2]. In pancreas, GAD65 is known to be a major autoantigen recognized by humoral and cellular immune components in both human and murine type 1 diabetes [3,4]. In testis, both GAD67 and GAD65 have been reported to present in Leydig cell, and GABA appeared to be linked to the regulation of steroid synthesis via GABA_A receptors [5,6]. *GAD65* and *GAD67* mRNAs have also been detected in testis germ cells [7,8], although the role of GABA in germ cell maturation and function remains to be established. Thus, further study of *GAD65* gene expression pattern is important to understand the physiological significance of GABAergic system in testis.

Previous researches proved that GAD65 function can be regulated by multiple mechanisms [9,10]. For example, in CNS, the majority of GAD65 exists as inactive apoenzyme, and its activity can be regulated by interaction with its cofactor pyridoxal phosphate [11]. At post-translational level, a more active and stable 58-kDa truncated GAD65 can be produced by cleaving at arginine 69 from NH₂-terminus of the full-length GAD65 [12]. However, whether and how *GAD65* gene expression is regulated at the transcriptional level remains unclear. Recent genome-wide analysis revealed that many genes possess more than one promoter, and alternative promoters play important roles in regulating gene expression [13]. As for rat *GAD65* gene, multiple transcription start sites (TSSs) transcribed from two independent TATA-less promoters were reported [14]; however, the functional significance of this finding is not known.

The regulation of gene expression is a critical, highly coordinated, and complex process, particularly in testis [15]. Whether and how *GAD65* gene expression is regulated during testis maturation is not known. In this study, rapid amplification of cDNA ends (RACE) technology was used to analyze the expression pattern of *GAD65* gene in post-natal Days 1 and 90 testis, from which two *GAD65* mRNA isoforms transcribed from distinct TSSs were characterized. We found that utilization of these TSSs is related to specific post-natal ages in the rat testis, indicating that *GAD65* gene expression was dynamically regulated by alternative promoters.

Materials and Methods

Materials

The following chemicals and reagents were purchased from Sigma (St Louis, MO, USA): rabbit anti-GAD65 antibody (G4913), mouse monoclonal anti-GAD65 antibody (GAD6), FITC-conjugated goat anti-rabbit IgG secondary antibody, HRP-conjugated goat anti-mouse, and goat anti-rabbit IgG secondary antibody. Trizol was from Invitrogen (Carlsbad, CA, USA). Smart RACE cDNA amplification kit was from Clontech (Mountain View, CA, USA). Oligo-dT primer, M-MLV reverse transcriptase, and pGEM T-Easy vector were from Promega (Madison, WI, USA). The synthetic peptide corresponding to the C-terminal region of human GAD65 (amino acids 514–530) was synthesized by GL Biochem Co. (Shanghai, China). All Sprague–Dawley rats used were obtained from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). Testes of different age were obtained from male rats. Ovary was obtained from 2-month-old female rats. Other tissues were obtained from 2-month-old male rats.

Rapid amplification of cDNA ends polymerase chain reaction

The RACE experiments were performed using the Smart RACE cDNA amplification kit. The 5' and 3' RACE-ready cDNAs were prepared using mRNA isolated from post-natal Day 1 or 90 rat testis according to the procedure supplied by the manufacturer. Nested PCR procedures were performed to increase the specificity. For GAD65 5' RACE, the external primers used in the two independent RACE experiments were 5'-GACTATGCTCTGACGTGAATGCGATG-3' and 5'-CTGGAAACATCTTATAGCGGGCAATG-3', and the internal was 5'-AAAGATTCCATCGCCAGAGCCTCC TG-3' and 5'-AGGTCACGCTCCCGCCGCTCTCTGC

T-3'. For GAD65 3' RACE, the external primer was 5'-C ATCGCATTACGTCAGAGCATAGTC-3' and the internal primer was 5'-CTTGCAAT GTTCGGCTCTCC TGGTCA-3'. The amplification products of 5' and 3' RACE were cloned into pGEM T-Easy vector and sequenced.

RNA extraction and RT–PCR

Total RNAs from Sprague–Dawley rat testis of different post-natal ages and various tissues were extracted using Trizol, and isolated according to the procedure supplied by the manufacturer. Transcription was performed according to the manufacturer's instructions. Conditions for PCR amplifications were as follows: initial denaturation at 94°C for 4 min; followed by 25 cycles of 30 s denaturation at 94°C; 30 s annealing at 60°C; and 1 min extension at 72°C. To amplify GAD65B, the forward primer (65B-f) was 5'-CGAGCACTGGCATACGCAGAC-3' and the reverse primer (65B-r) was 5'-GCCCTCTCCACACCGTTTCAG-3'. To analyze the total GAD65 expression, the forward primer (65-f) was 5'-GCCAGGCTCATCGCATTCA-3' and the reverse primer (65-r) was 5'-AGTCATTACAAATCT TGCCC-3'. To assure that the results are semi-quantified estimates, β -actin was co-amplified in the system and taken as the internal control.

Cell culture and transfection

Human embryonic kidney (HEK293) cells were maintained in a growth medium composed of Dulbecco's modified Eagle's medium supplemented with 10% FBS, 25 μ g/ml penicillin, and 100 μ g/ml of streptomycin. Cells were incubated at 37°C under an atmosphere of 5% CO₂. Transfection was performed at 20–24 h after subculturing with 10 μ g of plasmids (pcDNA3.1) containing the cDNAs of GAD65 and NH₂-terminal truncated GAD65 by calcium phosphate precipitation. The transfected cells were washed and incubated for 24–48 h before further study.

Western blotting analysis

Western blotting was performed as follows. To detect GAD65 expression in testis and brain tissues, lysates (60 μ g protein per load) prepared from rat testis at different developmental ages and rat cerebellum (1 μ g protein per load) were analyzed. To analyse GAD proteins expressed in HEK293 cells, cells were lysed with boiling sample buffer. The total proteins were then separated by SDS–PAGE and transferred to a nitrocellulose membrane, blocked with TBST buffer containing 5% fat-free

milk powder for 2 h at room temperature, and then incubated with the primary antibody overnight at 4°C. Rabbit anti-GAD65 antibody and mouse monoclonal anti-GAD65 antibody, GAD6, were diluted as 1:10 000 and 1:1000, respectively. For control experiments, the primary antibody was pre-incubated for 30 min at 4°C in blotting buffer with 100 ng/ml (final concentration) recombinant expressed full-length rat GAD65 protein or GAD65 (residues 514–530) peptide. After washing, the membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature followed by staining of the immunoreactive proteins using Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Immunohistochemical staining

Immunohistochemical localization of GAD was performed with cryostat sections of male Sprague–Dawley rat testis. The sections were fixed with 4% (w/v) paraformaldehyde, washed with PBS, and blocked with PBS plus 10% normal goat serum for 30 min at room temperature, then incubated with anti-GAD65 antibody (diluted 1:2000) for 1 h at room temperature. After thorough washing, the sections were incubated with FITC-conjugated goat anti-rabbit IgG secondary antibody, diluted as 1:300, for 45 min at 37°C. For the control, the first antibody was pre-absorbed with synthesized peptide used as immunogen. The sections were finally counterstained with DAPI to visualize the nuclei. The cellular localization of the staining was determined under a laser scanning confocal microscope (Leica TCS SP5).

Data analysis

All experiments were repeated at least three times. For semi-quantitative RT–PCR analysis, densitometric signals from individual bands were normalized to actin to correct for differences in gel loading of the mRNA changes, or of its selectivity, or both. The intensities of western blotting signals were quantified using ImageJ. Data are results from three independent experiments and are expressed as mean \pm SD.

Results

GAD65 transcripts in rat testis

Full-length *GAD65* gene sequences of post-natal Days 1 and 90 rat testes were characterized by RACE technology, respectively [Fig. 1(A)]. Two isoforms of GAD65 transcripts (GAD65A and GAD65B) transcribe from two

independent clusters of TSSs were identified [Figs. 1(B) and 2]. And repetitive experiments confirmed only these mRNA isoforms exist in post-natal testis. GAD65A transcribed from TSSs located \sim 100 bp upstream of the translation start site, under the regulation of the proximal promoter. GAD65B transcribed from TSSs located \sim 470 bp upstream of the translation start site, under the regulation of the distal promoter. Thus, except for the large 5'-UTR, GAD65B is identical to GAD65A. And both GAD65A and GAD65B possess the same open reading frame with previous reported GAD65 transcript, and encoding the 65 kDa full-length GAD65 protein.

Tissue expression pattern of GAD65 transcripts

Tissue distribution of the GAD65 transcripts was determined by RT–PCR using cDNAs of various rat tissues (Fig. 3). PCR using primer set that amplify both

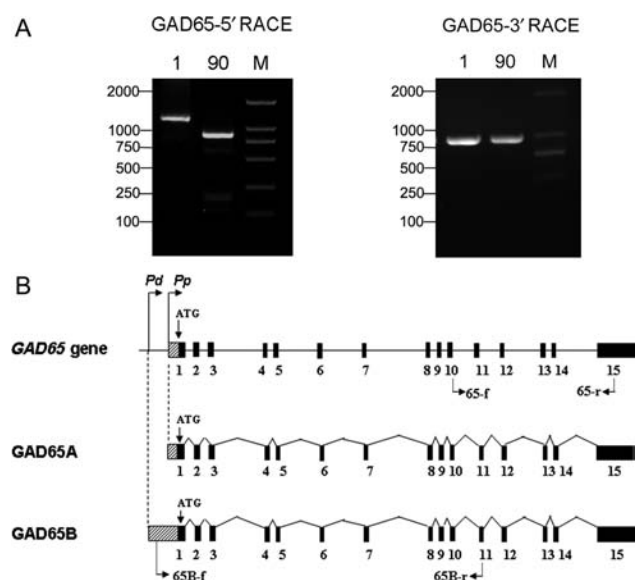


Figure 1 RACE analysis of rat testis GAD65 expression (A) The PCR products of the 5' and 3' RACE amplification of post-natal Days 1 and 90 testis GAD65 transcripts were visualized in 2% agarose gels. Lane M, DNA marker DL2000. (B) Schematic presentation of the full-length *GAD65* mRNAs identified by RACE method. The exons were numbered relative to their position in the *GAD65* gene. Two GAD65 mRNA isoforms, GAD65A and GAD65B, differing in the lengths of 5'-UTR were identified. GAD65A identified in post-natal Day 90 testis was transcribed from the proximal promoter (Pp). GAD65B identified in post-natal Day 1 testis was transcribed from the distal promoter (Pd). Compared with GAD65A, GAD65B possesses a much longer 5'-UTR (the hatched parts). The ATG codons for translation initiation of each mRNA isoform are marked by vertical arrows. Arrows below the exons stand for specific primers used in amplifying the GAD65 transcripts: 65B-f and 65B-r for analyzing GAD65B, 65-f and 65-r amplifying both GAD65A and GAD65B for analyzing total GAD65 expression.



Figure 2 Sequence analysis of rat GAD65 transcripts 5'-flanking region Two independent clusters of TSSs, from which GAD65B and GAD65A transcribed, were determined by 5' RACE-PCR. The TSSs are indicated by vertical arrows; the figures next to the arrows show the number of clones having the 5'-end at the corresponding nucleotide. Consensus sequences for putative transcription factor-binding sites are shown. Two overlapping near-consensus initiator elements (Inr) are present near the TSSs of GAD65A. The previous identified putative Sp1-binding site is also shown. The numbering is related to the translation start site (the asterisks).

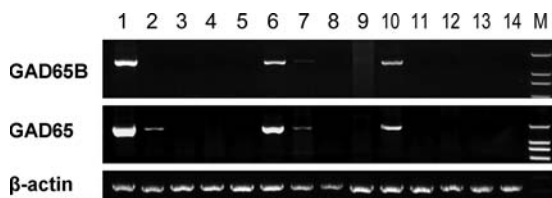


Figure 3 Semi-quantitative RT-PCR determination of GAD65 mRNA isoforms in various rat tissues The experiment was carried out by using the primer pairs described in Fig. 1(B). PCR products for GAD65B (GAD65B) and total GAD65 mRNA (GAD65) were visualized in 2% agarose gels. The tissues are numbered as follows: 1, brain; 2, heart; 3, kidney; 4, liver; 5, lung; 6, ovary; 7, pancreas; 8, skeletal muscle; 9, spleen; 10, testis; 11, uterus; 12, initial segment of epididymis; 13, corpus epididymis; 14, caudal epididymis; lane M, DNA marker DL2000.

GAD65A and GAD65B showed that GAD65 was expressed in multiple tissues, including brain, heart, ovary, pancreas, and testis. When amplified with GAD65B-specific primer, similar expression pattern was observed for GAD65B, but not detected in heart. Because GAD65A is identical to GAD65B but with shorter 5'-UTR, it is difficult to synthesize GAD65A-specific primer and analyze its expression pattern directly. However, GAD65A should be expressed in heart, which may explain the expression of GAD65 in this tissue.

Developmental expression of GAD65 transcripts in post-natal rat testis

To establish the developmental expression patterns of GAD65 transcripts during post-natal testis maturation,

semi-quantitative RT-PCR analysis was performed using rat testis cDNA obtained at different time periods after birth (1–90 days) (Fig. 4). Again, total GAD65 gene expression was analyzed by primer set that amplify both GAD65A and GAD65B. The results showed that GAD65 was expressed at all age points examined. From Days 1 to 30, its expression level was only slightly elevated and kept at relatively lower level. However, at Day 40, GAD65 expression was dramatically increased ~3 folds compared with that of 30-day-old rats. And this high expression level was maintained between Days 40 and 90 [Fig. 4(A)]. When amplified with GAD65B-specific primers, we found that its level was increased gradually from post-natal Days 1 to 15, thereafter sharply decreased, and almost undetectable on post-natal Day 40 [Fig. 4(B)]. Since without GAD65A-specific primer as mentioned above, its expression pattern cannot be analyzed directly.

Developmental expression of GAD65 protein in post-natal rat testis

To detect the protein, we first used a specific monoclonal anti-GAD65 antibody, GAD6. As anticipated, a 65-kDa band was found in brain. However, in testis, a 58-kDa band was found [Fig. 5(A)]. When pre-absorbed the antibody with the purified recombinant GAD65 protein, both bands disappeared [Fig. 5(B)]. Several lines of evidence indicate that 58-kDa band was the previously reported N-terminal truncated 58-kDa GAD65 resulted from post-translational process [12]. To verify these, HEK293 cells were transfected with transcripts of

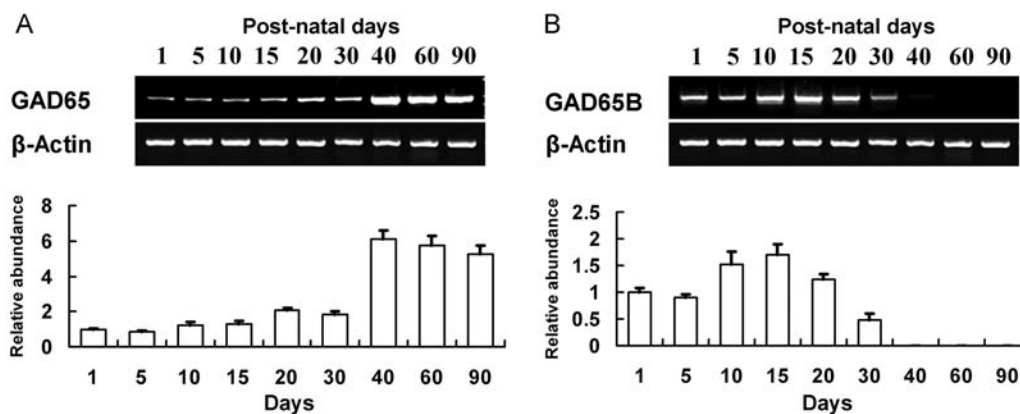


Figure 4 Semi-quantitative RT-PCR determination of *GAD65* mRNA isoforms in testis from 1 to 90 post-natal day rats using specific primer pairs described above. β -Actin was co-amplified and taken as the internal control. (A) Developmental expression of total *GAD65* mRNA. Signals were corrected with β -actin and normalized to the first day of age, which was arbitrarily designated as 1. Data are results from three independent experiments and are expressed as mean \pm SD. (B) Developmental expression of *GAD65B*. The relative transcription level of *GAD65B* at each post-natal day was analyzed as above.

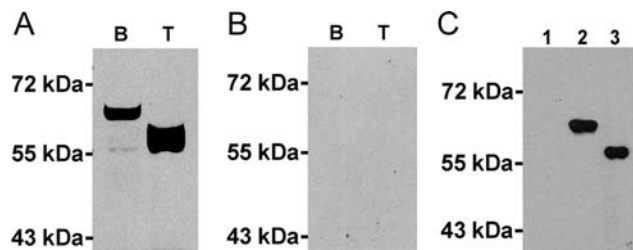


Figure 5 Western blotting analysis of *GAD65* expression in rat testis (A) Immunoblotting performed with monoclonal anti-*GAD65* antibody, *GAD6*. In the rat cerebellum (lane B), a single moderately stained 65-kDa band and a faintly stained 58-kDa band were detected, whereas in the adult testis (lane T), only a single 58-kDa band was visualized. (B) The control for the monoclonal *GAD6* antibody. No band was detected when the monoclonal antibody was pre-absorbed with the purified recombinant rat *GAD65* protein. (C) Immunoblotting analysis of *GAD65* forms transfected in HEK293 using a polyclonal anti-*GAD65* antibody. Lane 1, cell transfected with the vacant vector; lane 2, *GAD65*; lane 3, *GAD65* deleted of NH_2 -terminal 69 amino acids.

GAD65 or NH_2 -terminal 69 amino acids deleted *GAD65*, and analyzed by immunoblotting with a polyclonal anti-*GAD65* antibody, which indicated molecular weight identical to the respective testis form [Fig. 5(C)]. Immunoblotting was then employed to further clarify the developmental changes in the expression of *GAD65* protein using this polyclonal anti-*GAD65* antibody. The 58-kDa band was consistently detected at each post-natal day, which showed an age-dependent increase [Fig. 6(A)]. Moreover, a faint, yet clear 65-kDa band corresponding to the one in cerebellum was also detected in all the testis samples [Fig. 6(A), arrow]. The specificity of the antibody was verified by the absence of

staining when pre-absorbed the antibody with the synthesized peptide utilized for immunogen [Fig. 6(B)].

Localization of *GAD65* protein in rat testis

The location of *GAD65* in adult rat testis was further studied by immunofluorescence method. In post-natal Day 1 testis, *GAD65* immunosignal located at the Leydig cells [Fig. 7(A), red arrow], whereas in post-natal Day 90 testis, *GAD65* immunoreactivity was detected in primary spermatocytes [Fig. 7(B), yellow arrow] and the elongated spermatids [Fig. 7(B), red arrow]. No staining occurred when the primary antibody was pre-absorbed with synthesized peptide used as immunogen for anti-*GAD65* antibody [Fig. 7(C)]. The sections were further counterstained with DAPI to visualize the nuclei [Fig. 7(D–F)]. And the corresponding merged pictures are also shown [Fig. 7(G–I)].

Discussion

GAD is the key enzyme responsible for the synthesis of GABA, the main inhibitory neurotransmitter in adult mammalian brain. Two isoforms of *GAD*, *GAD65* (65 kDa) and *GAD67* (67 kDa), are found in mammalian CNS. The present findings revealed that GABA-synthesizing enzyme *GAD65* also existed in post-natal testis, and its expression was dynamically regulated at multiple levels during post-natal testis maturation.

Variant transcripts produced by alternative TSSs are a frequent occurrence in the testis, which is presumably due to the activation of alternative promoters [16,17].

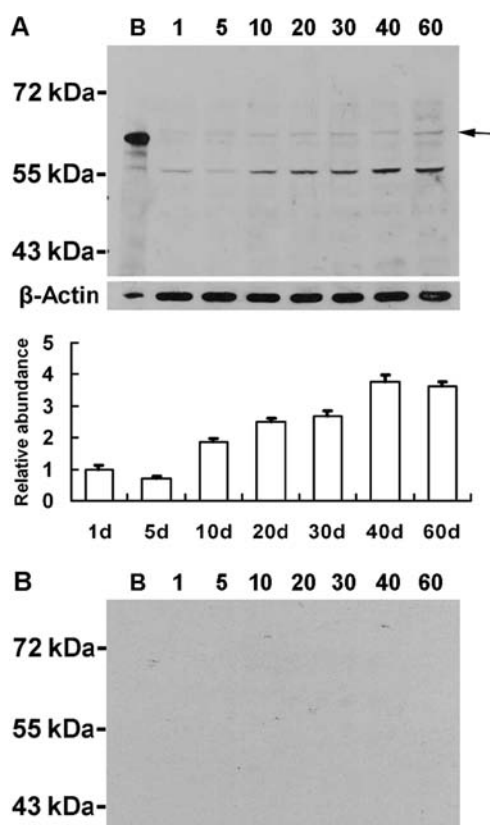


Figure 6 Western blotting analysis of GAD65 protein expression during post-natal testis maturation (A) Immunoblotting was performed using the polyclonal anti-GAD65 antibody. The number represented post-natal days. With rat cerebellum (lane B), a 65-kDa band was detected. In rat testis, a major band of 58-kDa was consistently detected at each developmental age. Moreover, a faint, yet clear 65-kDa band corresponding to the one in cerebellum was also detected in all the testis samples (A, arrow). Bars represent relative level of total GAD65 protein at each age point. Signals for GAD65 protein were standardized to the actin signal, and then normalized to the first day of age, which was arbitrarily designated as 1. Each bar represents the average of relative band intensity from three experiments \pm SEM. (B) The control for the polyclonal anti-GAD65 antibody. No specific band was detected in each sample analyzed above when pre-absorbed the antibody with the synthesized peptide utilized for immunogen.

Previous report showed that rat *GAD65* gene possesses two active TATA-less promoters. The distal promoter directs transcription from TSSs at or around -396 bp upstream of translation start site, whereas the proximal promoter directs transcription from within the -101 to -87 region [14]. Here, TSSs for *GAD65* genes in rat post-natal Days 1 and 90 testis were separately mapped with 5' RACE methods. By analyzing multiple clones of the 5' RACE product, we found that in post-natal Day 1 testis, the TSSs located up to -484 bp upstream of the

translation start site. However, in post-natal Day 90 testis, TSSs mainly located within -102 bp. The resulting difference in sequence of the 5' regions of the mRNAs may correlate with the specific translational regulation that occurs in testis [18]. And there are well-documented examples of post-transcriptional regulation that results in a critical delay between production of certain mRNAs and their subsequent translation [19,20]. This is likely, as we observed that total *GAD65* mRNA level increased ~ 3 folds from post-natal Days 30 to 40 [Fig. 4(A)], whereas GAD65 protein increased only 40% at most [Fig. 6(A)].

Gene expression often shows stage-specific change during post-natal testis maturation [15,21]. Here, our RT-PCR results showed that GAD65 was expressed at each day examined. Total expression level of *GAD65* mRNA was elevated slightly from Days 1 to 30; nevertheless from Day 40, its level dramatically increased ~ 3 folds [Fig. 4(A)]. Age-dependent increase in protein levels were also observed by western blotting analysis [Fig. 6(A)]. Since GAD65B expression level was down-regulated after post-natal Day 20, the elevation of total GAD65 mRNA and protein level after post-natal Day 20 should be due to the activation of the proximal promoter which transcribes GAD65A. Previous researches also showed that the minor, distal promoter was responsible for $<20\%$ of all initial events. And reporter gene analysis demonstrated that the distal promoter could be omitted without any significant reduction in activity [14]. Thus, the two tandemly arranged promoters probably have different strengths. Therefore, the use of the more active proximal promoter may account for the significant increasing of GAD65 transcription level from post-natal Day 40 [Fig. 4(A)]. All these observations indicated that *GAD65* gene expression is regulated by alternative promoters at the transcription level.

Although GAD67 has long been found in testis, whether mammalian testis also possesses GAD65 is quite controversial. Tillakaratne *et al.* [1] failed to detect GAD65 expression by the immunoprecipitation method. Both *in situ* hybridization method and study of transgenic mouse with GAD65 promoter showed that *GAD65* mRNA was confined primarily to the spermatocytes and spermatids of seminiferous tubule [7,22]. Here, by immunoblotting, a 58-kDa band and a faint 65-kDa band corresponding to the full-length GAD65 were detected by anti-GAD65 antibodies [Figs. 5(A) and 6(A)]. Because the molecular weight of IgG heavy chain is ~ 55 – 58 kDa, the immunoprecipitation method is not suitable to detect the 58-kDa GAD65 in the testis because the

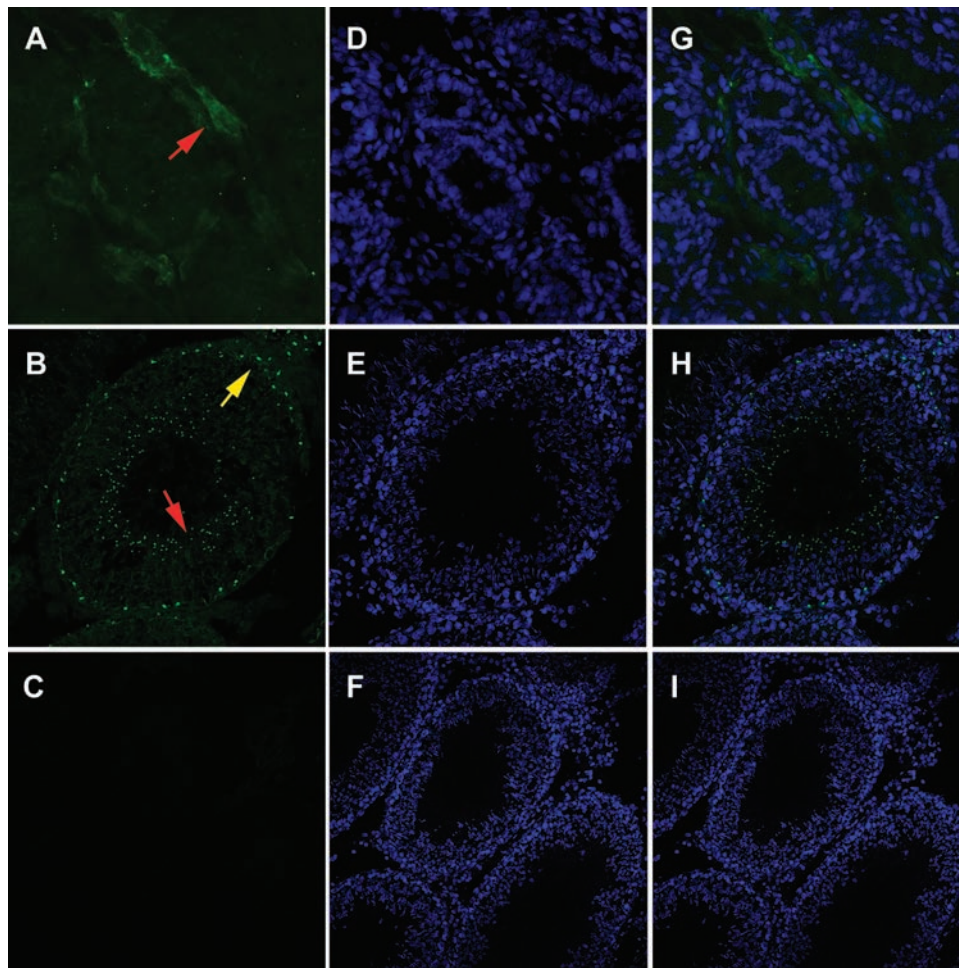


Figure 7 Localization of GAD65 in rat testis by immunofluorescence microscopy In post-natal Day 1 testis, GAD65 immunostaining visible as green fluorescence occurred in the Leydig cells (A, red arrow). In post-natal Day 90 testis, GAD65 immunostaining occurred in the spermatocytes (B, yellow arrow) and testicular spermatids (B, red arrow). When the first antibody was pre-absorbed with immunogenic peptide, no staining occurred (C). Nuclei were stained blue with DAPI (D–F); the corresponding merged pictures (G–I).

two proteins will not be easily distinguished by SDS–PAGE. This may explain why Tillakaratne *et al.* [1] failed to detect the 58-kDa GAD65. The existence and location of GAD65 in post-natal Days 1 and 90 testis was further studied by our immunofluorescence experiment. The results showed that in post-natal Day 1 testis, it was located in the Leydig cells; whereas in post-natal Day 90 adult testis, it was located in spermatocytes and elongated spermatids. The location difference indicates that *GAD65* gene was possibly expressed in different cell types during post-natal testis maturation.

Here, by immunoblotting, the same 58-kDa band was consistently recognized by a monoclonal and a polyclonal anti-GAD65 antibody in post-natal testis [Figs. 5(A) and 6(A)]. As epitope locations of both antibodies are mapped in the C-terminal of GAD65, it can be inferred that the testis GAD65 C-terminus is intact, and

truncation takes place in the NH₂-terminus. Previous research showed that 58-kDa GAD65 could be produced by cleavage at arginine 69 from NH₂-terminus of the full-length GAD65 both *in vitro* and *in vivo* [12,23]. And the resulted truncated GAD65 is more stable and active than the full-length form [12]. In brain, calpain, a calcium-dependent cysteine protease, is activated upon neuronal stimulation and could be responsible for the conversion of full-length GAD65 to truncated GAD65 [23]. Since calpain is an active enzyme in testis [24], the same proteolytic mechanism may take place which results in the 58-kDa GAD65 in testis. And the weak 65-kDa band should be the unproteolyzed full-length GAD65 [Fig. 6(A), arrow]. Although its level is relatively low, full-length GAD65 may also play an important role in testes GABA synthesis. The NH₂-terminus of GAD65 has been reported to be involved in membrane

anchoring of GAD65 and considered to be the site that confers and regulates the enzymatic activity [9,12,25]. Therefore, in rat testis, GAD65 function should be regulated at the post-translational level, and the resulted truncated GAD65 may play a role different from the full-length GAD65.

GABA is reported to modulate neuron progenitor cell proliferation, neuron differentiation, migration, and survival of neural cells during early CNS development [26–29]. GABA concentration in rat testis was measured by Erdo *et al.* [30] to be ~ 50 nmol/g wet tissue. GAD activity was detected in spermatocytes and spermatids of rat testis [1]. These observations indicated that testis GAD should be functional. Previous studies also showed that GABA receptors (GABA_A, GABA_B, and GABA_C), and GABA transporter (GAT-1) exist in rat testis Leydig cells and testicular germ cell [6,31–34]. And GABA appeared to be linked to the regulation of steroid synthesis by Leydig cells via GABA_A receptors [5,6]. Moreover, GABA may also play an as yet unrecognized role in the development of Leydig cells during testis maturation [35]. All these observations implied that a GABAergic system may function in testis. Here, we found that GAD65 was consistently expressed at each post-natal day examined. Since both GAD67 and GAD65 may participate in GABA synthesis, and the expression pattern of GAD67 during post-natal testis maturation was not clear, we cannot clarify to what level GAD65 participates in the GABA synthesis. Thus, further study of the expression pattern of GAD67 was required. In conclusion, our results showed that *GAD65* gene expression has a distinct expression pattern during post-natal testis maturation, which indicated that it was regulated by multiple mechanisms. Thus, by dynamic regulation of *GAD65* gene expression, GABA may precisely modulate testis maturation through autocrine or paracrine pathways.

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References

- 1 Tillakaratne NJ, Erlander MG, Collard MW, Greif KF and Tobin AJ. Glutamate decarboxylases in nonneural cells of rat testis and oviduct: differential expression of GAD65 and GAD67. *J Neurochem* 1992, 58: 618–627.
- 2 Reetz A, Solimena M, Matteoli M, Folli F, Takei K and De Camilli P. GABA and pancreatic beta-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO J* 1991, 10: 1275–1284.
- 3 Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M and Folli F, *et al.* Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 1990, 347: 151–156.
- 4 Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting GS, Robinson P and Atkinson MA, *et al.* Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 1993, 366: 69–72.
- 5 Ritta MN and Calandra RS. Occurrence of GABA in rat testis and its effect on androgen production. *Adv Biochem Psychopharmacol* 1986, 42: 291–297.
- 6 Geigerseder C, Doepner R, Thalhammer A, Frungieri MB, Gamel-Didelon K, Calandra RS and Kohn FM, *et al.* Evidence for a GABAergic system in rodent and human testis: local GABA production and GABA receptors. *Neuroendocrinology* 2003, 77: 314–323.
- 7 Faulkner-Jones BE, Cram DS, Kun J and Harrison LC. Localization and quantitation of expression of two glutamate decarboxylase genes in pancreatic beta-cells and other peripheral tissues of mouse and rat. *Endocrinology* 1993, 133: 2962–2972.
- 8 Persson H, Peltto-Huikko M, Metsis M, Soder O, Brene S, Skog S and Hokfelt T, *et al.* Expression of the neurotransmitter-synthesizing enzyme glutamic acid decarboxylase in male germ cells. *Mol Cell Biol* 1990, 10: 4701–4711.
- 9 Wei J and Wu JY. Post-translational regulation of L-glutamic acid decarboxylase in the brain. *Neurochem Res* 2008, 33: 1459–1465.
- 10 Curran-Rauhut MA and Petersen SL. Regulation of glutamic acid decarboxylase 65 and 67 gene expression by ovarian steroids: identification of two functionally distinct populations of GABA neurons in the pre-optic area. *J Neuroendocrinol* 2002, 14: 310–317.
- 11 Martin DL, Martin SB, Wu SJ and Espina N. Regulatory properties of brain glutamate decarboxylase (GAD): the apoenzyme of GAD is present principally as the smaller of two molecular forms of GAD in brain. *J Neurosci* 1991, 11: 2725–2731.
- 12 Wei J, Jin Y, Wu H, Sha D and Wu JY. Identification and functional analysis of truncated human glutamic acid decarboxylase 65. *J Biomed Sci* 2003, 10: 617–624.
- 13 Ayoubi TA and Van De Ven WJ. Regulation of gene expression by alternative promoters. *FASEB J* 1996, 10: 453–460.
- 14 Skak K and Michelsen BK. The TATA-less rat GAD65 promoter can be activated by Sp1 through non-consensus elements. *Gene* 1999, 236: 231–241.
- 15 Eddy EM. Regulation of gene expression during spermatogenesis. *Semin Cell Dev Biol* 1998, 9: 451–457.
- 16 Kleene KC. Patterns, mechanisms, and functions of translation regulation in mammalian spermatogenic cells. *Cytogenet Genome Res* 2003, 103: 217–224.
- 17 Davuluri RV, Suzuki Y, Sugano S, Plass C and Huang TH. The functional consequences of alternative promoter use in mammalian genomes. *Trends Genet* 2008, 24: 167–177.
- 18 Hughes TA. Regulation of gene expression by alternative untranslated regions. *Trends Genet* 2006, 22: 119–222.
- 19 Yang J, Medvedev S, Reddi PP, Schultz RM and Hecht NB. The DNA/RNA-binding protein MSY2 marks specific transcripts for

- cytoplasmic storage in mouse male germ cells. *Proc Natl Acad Sci USA* 2005, 102: 1513–1518.
- 20 Braun RE. Temporal control of protein synthesis during spermatogenesis. *Int J Androl* 2000, 23: 92–94.
- 21 Choi E, Lee J, Oh J, Park I, Han C, Yi C and Kim do H, *et al.* Integrative characterization of germ cell-specific genes from mouse spermatocyte UniGene library. *BMC Genomics* 2007, 8: 256.
- 22 Makinae K, Kobayashi T, Shinkawa H, Sakagami H, Kondo H, Tashiro F and Miyazaki J, *et al.* Structure of the mouse glutamate decarboxylase 65 gene and its promoter: preferential expression of its promoter in the GABAergic neurons of transgenic mice. *J Neurochem* 2000, 75: 1429–1437.
- 23 Wei J, Lin CH, Wu H, Jin Y, Lee YH and Wu JY. Activity-dependent cleavage of brain glutamic acid decarboxylase 65 by calpain. *J Neurochem* 2006, 98: 1688–1695.
- 24 Ben-Aharon I, Brown PR, Etkovitz N, Eddy EM and Shalgi R. The expression of calpain 1 and calpain 2 in spermatogenic cells and spermatozoa of the mouse. *Reproduction* 2005, 129: 435–442.
- 25 Shi Y, Veit B and Baekkeskov S. Amino acid residues 24–31 but not palmitoylation of cysteines 30 and 45 are required for membrane anchoring of glutamic acid decarboxylase, GAD65. *J Cell Biol* 1994, 124: 927–934.
- 26 Barbin G, Pollard H, Gaiarsa JL and Ben-Ari Y. Involvement of GABAA receptors in the outgrowth of cultured hippocampal neurons. *Neurosci Lett* 1993, 152: 150–154.
- 27 Behar TN, Schaffner AE, Colton CA, Somogyi R, Olah Z, Lehel C and Barker JL. GABA-induced chemokinesis and NGF-induced chemotaxis of embryonic spinal cord neurons. *J Neurosci* 1994, 14: 29–38.
- 28 Liu J, Morrow AL, Devaud L, Grayson DR and Lauder JM. GABAA receptors mediate trophic effects of GABA on embryonic brainstem monoamine neurons in vitro. *J Neurosci* 1997, 17: 2420–2428.
- 29 Ikeda Y, Nishiyama N, Saito H and Katsuki H. GABAA receptor stimulation promotes survival of embryonic rat striatal neurons in culture. *Brain Res Dev Brain Res* 1997, 98: 253–258.
- 30 Erdo SL, Nemet L and 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.
- 31 Hu JH, He XB, Wu Q, Yan YC and Koide SS. Subunit composition and function of GABAA receptors of rat spermatozoa. *Neurochem Res* 2002, 27: 195–199.
- 32 Li S, Zhang Y, Liu H, Yan Y and Li Y. Identification and expression of GABAC receptor in rat testis and spermatozoa. *Acta Biochim Biophys Sin* 2008, 40: 761–767.
- 33 He X, Zhang Y, Yan Y, Li Y and Koide SS. Identification of GABABR2 in rat testis and sperm. *J Reprod Dev* 2003, 49: 397–402.
- 34 Hu JH, He XB and Yan YC. Identification of gamma-aminobutyric acid transporter (GAT1) on the rat sperm. *Cell Res* 2000, 10: 51–58.
- 35 Doepner RF, Geigerseder C, Frungieri MB, Gonzalez-Calvar SI, Calandra RS, Raemsch R and Fohr K, *et al.* Insights into GABA receptor signalling in TM3 Leydig cells. *Neuroendocrinology* 2005, 81: 381–390.