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Identification of γ-aminobutyric acid transporter (GAT1) on the rat sperm

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ABSTRACT

Some recent studies indicated that GABAergic system is involved in mammalian sperm acrosome reaction (AR), but direct evidence pertaining to the expression of gat1 in mammalian sperm is not yet demonstrated. In this study, we evaluated the presence of 67kDa GAT1 protein and mRNA in rat testis by Western blotting and reverse transcription-polymerase chain reaction. Meanwhile, immunohistochemical and immunofluorescent analyses also identified GAT1 protein on the elongated spermatid and sperm. These results indicated that rat testis is a novel site of gat1 expression. Further studies should be taken to explore the role of GAT1 protein on sperm acrosome reaction.

Key words: *Gat1, rat testis, sperm, expression.*

INTRODUCTION

g-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the vertebrate central nervous system (CNS)[1],[2]. Outside the CNS, many peripheral tissues have also been found to have GABAergic system[3]. Moreover, the rat oviduct has been found to be more than twice as rich in GABA content than the brain[4].

The acrosome reaction (AR), a fundamental process in mammalian fertilization, can be viewed as a unique form of exocytosis. The AR releases acrosomal contents (including hydrolytic enzymes), which ensure sperm penetration of the egg zona pellucida, an acellular glycoprotein envelope overlying the egg plasma membrane, and fusion of the egg and sperm plasma membrane. In order to respond to physiological AR initiators, mam-

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malian sperm must first undergo cellular changes called capacitation[5]. Erdö and Wekerle [6] found that the sperm membranes appeared to have GABA-specific binding sites. GABA at relatively low concentrations (0.5 μM) may mimic the effect of progesterone, a putative initiator of AR in vivo, increasing the fraction of acrosome-reacted cells in capacitated mouse spermatozoa[7]. Aanesen et al[8] suggested a possible existence of GABA-specific transport protein in human spermatozoa. Meanwhile, these authors also demonstrated that progesterone could stimulate GABA uptake in human spermatozoa[9].

Amino acid neurotransmitter transporters play a key role in the regulation of extracellular amino acid concentrations and termination of neurotransmission in the CNS[10]. GABA transporters are members of a large family characterized structurally by a homology in amino acid sequence and functionally by their co-transporting of Na^+ and Cl^- across the cell membrane[11]. It has been suggested as early as 1982 that in fowl spermatozoa there existed a carrier-mediated transport of GABA[12], and the presence of glutamate transporter, another amino acid transporter, was also reported recently on boar spermatozoa[13].

The aim of the present study is to identify directly whether GABA transporter-1, an important member of GAT family, is present in the rat testis, and ascertain its subcellular localization, by examining the expression of GAT1 protein and mRNA using the Western-blot, RT-PCR, immunohistochemical and immunofluorescent techniques.

MATERIALS AND METHODS

Materials

The following chemicals and reagents were purchased from Sigma Chemical Co.: DAB, Fraction V bovine serum albumin, EDTA, β-mercaptoethanol, benzamidine, Triton X-100, FITC-goat anti-rabbit IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase. Trizol from GIBCO. PolyAtract kit from Promega and ECL reagent from Amersham Pharmacia Biotech.

Rabbit anti mouse GAT1 antiserum, was kindly provided by Dr. Guo Li-He of this Institute.

Rat, Wistar male, 90 days of age.

Methods

Preparation of sample

Rat testis and brain were freshly excised, and live spermatozoa were collected from epididymes by swim-up method as previously described[14]. For Western blot, tissues or cells were homogenized in disruption buffer (10 mM Tris, pH 8.3, 1 mM EDTA, 1% Triton X-100, 1mM PMSF, 5 mM benzamindine), then vortexed at 4°C for 1 h, sonicated for 2 min and centrifugated at 10,000 rpm for 30 min. The supernatants were collected and dialyzed in 5 mM NH_4HCO_3 overnight at 4°C, lyophilized and stored at -20°C for later use. For RT-PCR, fresh tissues were immediately homogenized in Trizol.

Western-blot analyses

Samples were separated on a 12% polyacrylamide gel in the presence of SDS according to the procedure of Laemmli[15]. Fifty micrograms protein was loaded for each lane. After electrophoresis, protein was transferred onto a nitrocellulose filter. The membrane was blocked

with 10% dry milk in TBS, incubated with anti-GAT1 antiserum diluted 1:200 in TBST (TBS containing 0.1% Triton X-100), then washed three times with TBS and exposed to goat anti-rabbit IgG labeled with horseradish peroxidase (1:1000 dilution) at room temperature for 1 h. After washing three times with TBS, reaction was visualized using ECL reagents according to the manufacturer's instructions. As a positive control, rat brain extracts were used.

RT-PCR

Total testis RNA was extracted using Trizol according to the procedure described by the manufacturer. RNA integrity was tested by agarose gel electrophoresis in the presence of 2.2 M formaldehyde. Then mRNA was isolated using PolyAtract Kit from Promega. First strand cDNA was synthesized using Superscript Preamplification System from GIBCO. The blank for each RT reaction consisted of all of the reagents, with water substituted for RNA.

GAT1 primers: 5'-GCTTGTGTATTTCTGCATCTGG-3'(corresponding to GAT1 seq. 818-839) and 5'-CATGGAGGACAGAGCTAGGA-3'(corresponding to GAT1 seq. 1751-1732).

PCR was carried out in a programmable thermal cycler. Samples were first denatured at 94°C for 4 min, and then amplifications were carried out: 1 min at 94°C, 1 min at 58°C, 1 min at 72° C for 30 cycles followed by a final 10 min at 72°C. A blank was prepared using the corresponding RT blank. PCR solution was fractionated by electrophoresis in 1% agarose gel. Gels were stained with ethidium bromide, destained, and photographed.

Immunohistochemistry

Immuohistochemical staining was performed by an indirect peroxidase-conjugated method on 6 mm-thick paraffin sections of testis tissue which had been previously fixed in Carnoy's solution. Sections were deparaffinized in xylene and rehydrated to water through a graded alcohol series. After rinsed in TBST, sections were incubated with anti-GAT1 antiserum (1:200 dilution in TBST) or normal nonimmune rabbit serum as a negative control for at least 12 h at 4°C. Slides were then washed four times in TBST, followed by applying to peroxidase conjugated goat anti-rabbit IgG-horseradish diluted 1:1000 in TBS. The slides were then washed three times in TBS, and incubated in diaminobenzidine tetrahydrochloride in TBS with 0.01% hydrogen peroxide. The reaction was stopped by washing the sections in distilled water, and slides were mounted and observed under a light microscope. Endogenous peroxidase activity was quenched by incubating rehydrated tissue sections in methanol containing 1% hydrogen peroxide for 30 min at room temperature.

Immunofluorescence

For indirect immunofluorescent studies of GAT1 localization, epididymal spermatozoa were released into PBS and were allowed to disperse for 5 min before being collected by centrifugation at 7,500 g (20 min at room temperature), cells were resuspended in PBS to a final concentration of 5 ~ 6 × (10^6/ml. Sperm cells were fixed in 4% formaldehyde (W:V) for 30 min followed by blocking with 3% BSA in PBS for 30 min and then incubated with anti-GAT1 antiserum diluted 1:200 in PBS containing 1% BSA at room temperature for 2 h. After further three washings in TBS for 5 min each, incubation with FITC-goat anti-rabbit IgG was carried out under room temperature for 1 h. Unbound antibody was removed from sperm suspensions via 3 ~ 5 min washing in PBS containing 1% BSA. Sperm were then smeared on slides and observed by epifluorescence microscopy. As a negative control, antiserum was replaced by normal nonimmune rabbit serum.

RESULTS AND DISCUSSION

It has been found that GABA, the major amino acid neurotransmitter in the mammalian central nervous system (CNS), exists and plays an important physiological

role in many systems outside the CNS[3]. Results from other studies indicated a role for GABA in mammalian fertilization process[13],[16]. Aanesen et al in 1996 has found the presence of GABA uptake in human sperm and suggested that GABA transporter may be involved in regulating the action of GABA on sperm[8], [9]. The present study was designed to obtain more direct evidence for the presence of GAT1 in the rat testis and sperm. Our results were consistent with and extended those previous findings.

Identification of GAT1 and corresponding mRNA in rat testis and sperm

The presence of GAT1 protein in mature rat testis was assessed by Western blot analysis, by demonstrating the appearance of a characteristic 67 KDa band in total protein extracts of testis and sperm proteins were shown in Fig 1, lane 2 and 3. It is obvious that the 67 KDa protein detected in testis and sperm extracts was not different in size from the immunoreactive GAT1 protein found in rat spinal cord (Fig 1, lane 1)[17]. It is intersting to note that the staining intensity of sperm protein is much stronger than protein extracts from testis. To determine whether the presence of GAT1 protein is reflected in the level of mRNA, mRNA extracted from rat testis was examined by RT-PCR. As shown in Fig 2, when amplification was carried out in the presence of primers which designed according to the sequence of the rat brain gat1 cDNA, an intense 979 bp band corresponding in size to the brain gat1 cDNA product was obtained from the cDNA of testis specimen examined. Western blot and RT-PCR analyses indicated the presence of both GAT1 protein and corresponding mRNA in rat testis. In CNS, the majority of neurons expressing gat1 mRNA also contained immunoreactive glutamate decarboxylase (GAD) which can transform glutamate into GABA in vivo. Recently, GAD immunoreactivity was also observed in testis[18]. Our results thus confirm the postulation and prove directly the presence of GAT1 in rat testis and sperm at molecular level.

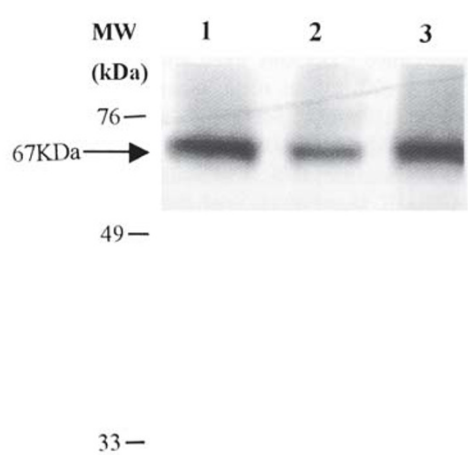

Fig 1.
Western immunoblot showing the presence of GAT1 protein in uncapacitated rat sperm protein extracts, rat whole testis and rat spinal extracts after SDS-PAGE (50 μ g protein was loaded in each lane).
lane 1. rat spinal cord extracts;
lane 2. total testis extracts;
lane 3. rat sperm protein extracts.
Blots were incubated with rabbit anti-GAT1 antiserum followed by treating with goat anti-rabbit IgG conjugated to HRP. Number refers to molecular mass (kDa) based on protein standards.

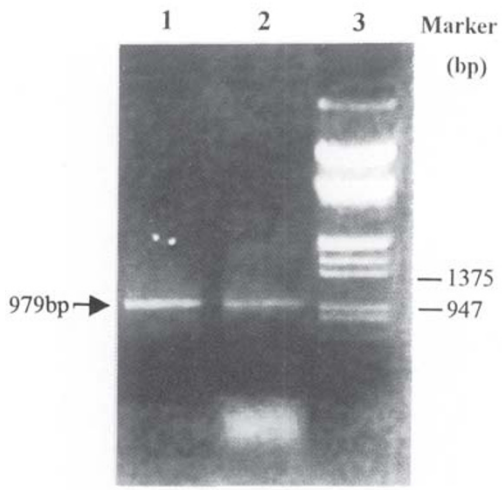

Fig 2.
RT-PCR analysis of GAT1 mRNA in rat testis and spinal RNA extracts.
Lane 1. RT-PCR product of spinal RNA extracts;
lane 2. RT-PCR product of whole testis RNA extracts;
lane 3. Marker (λ /EcoRI+HindIII).
Number indicates the molecular weight of the specific bands.

Cellular localization of GAT1 in rat testis and sperm

Localization of GAT1 protein in the testis was evaluated by immunohistochemical staining and immunofluorescence. As shown in Fig 3, obvious positive immunoreactivity was observed in the elongated spermatids and spermatozoa region in the sections of rat

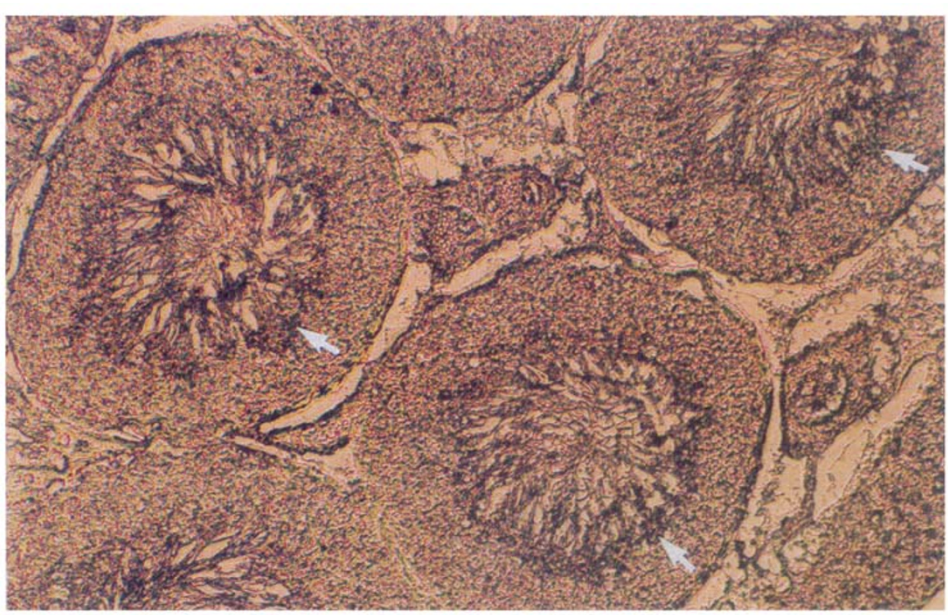

Fig 3.
Immunohistochemical localization of GAT1 in 90-day-old rat testis. (A) Section sincubated with rabbit anti-GAT1 antiserum followed by incubated with HRP-labeled second antibody. 200 ×; Arrows indicate the positive staining on the elongated spermatids and spermatozoa.

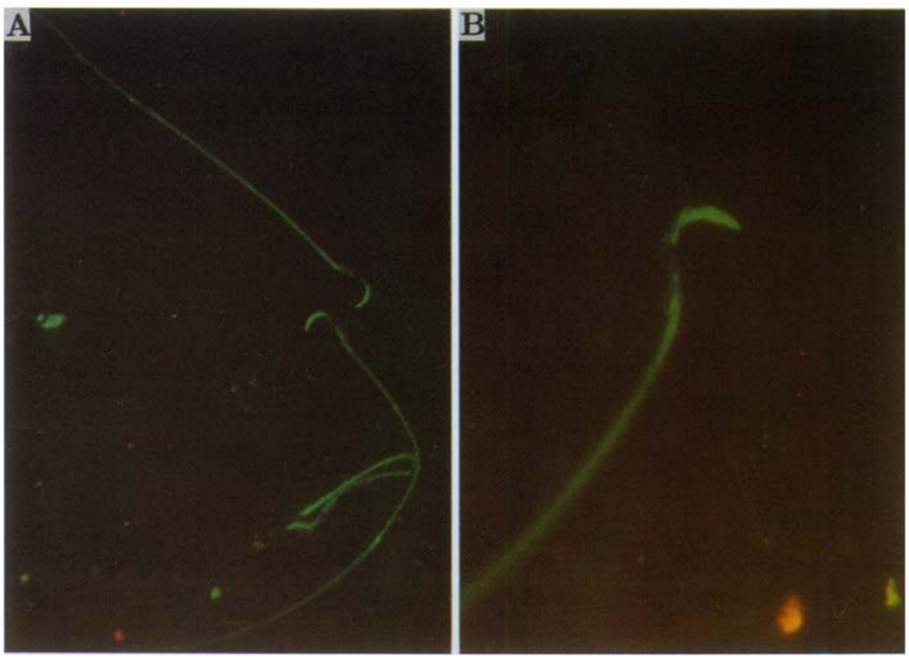

Fig 4.
Indirect immunofluorescence localization of the GAT1 in rat sperm. Paraformaldehyde-fixed, uncapacitated rat sperms were incubated with rabbit anti-GAT1 antiserum followed by overlaying with FITC-labeled goat anti-rabbit IgG. Spermatozoa were photographed under U.V. Sample incubated with rabbit anti-GAT1 antiserum. (A), 400 ×; (B), 1000 ×.

testis, and only elongated spermatids and spermatozoa displayed the strong staining, but not in spermatocytes, spermatogonia, sertoli cells and leydig cells. Staining was absent when the primary antibody was substituted by nonimmune serum (data not shown). Meanwhile, when sperm were stained with the anti-GAT1 antiserum after fixed in 4% formaldehyde, about 90% of them exhibited strong flourescence in the whole head except for the equatorial region, as illustrated in Fig 4. In the flagellum, staining could be also observed. Omission of the primary and/or secondary antibody abolished the sperm immunoreactivity entirely, (data not shown) just like in the controls in which primary antibody was replaced by normal rabbit nonimmune serum]. This observation was in agreement with the result of immunohistochemical staining shown in Fig 3. Since only the elongated spermatids and spermatozoa in testis express GAT1 protein, it is evident that the difference of the staining intensity between lane 2 and 3 in Fig 1 is due to their difference in the relative GAT1 content in protein extracts. The existence of GAT1 can lead to the membrane depolarization which has been demonstrated by other studies[19], [20]. Meanwhile, membrane depolarization is also important for the initiation of the sperm AR[21], so we postulate a possible role of GAT1 in sperm AR.

The present report provides the first direct evidence for GAT1 in sperm and supports the earlier suggestion that this transporter is involved in an essential fertilization event-AR. Further studies will be needed to investigate what exact role the GAT1 plays in the mammalian reproduction system and fertilization process.

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