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Identification of $\gamma 1$ subunit of GABAA receptor in rat testis

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ABSTRACT

The isoform type of g subunits of $GABA_A$ receptor is a molecular determinant of its pharmacological characteristics. At present, the existence of $GABA_A$ receptor in mammalian sperm is still a controversy. By using degenerate primers designed according to highly conserved region in all three γ ($\gamma 1$, $\gamma 2$ and $\gamma 3$) subunits cloned in rat brain, we performed reverse transcription polymerase chain reaction (RT-PCR) to examine the expression pattern of g subunits of $GABA_A$ receptor in rat testis. Only one 370 bp fragment was obtained from RT-PCR in rat testis and sequencing results showed that it represented g1 subunit, but not $\gamma 2$ or $\gamma 3$ subunit. Using the cloned \fragment as probe, a 3.8 kb transcript which in size as same as $\gamma 1$ subunit in rat brain was detected in rat testis mRNA by performing Northern blot assay. Furthermore, results of in situ hybridization assay confirmed that $\gamma 1$ subunit was expressed in round spermatids and spermatozoa, maybe also in secondary spermatocyte. These evidences proved that $\gamma 1$ subunit of $GABA_A$ receptor is exclusively expressed in rat testis and this feature may be the structural basis of the specific function of $GABA_A$ receptors in sperm acrosome reaction.

Key words: *GABAA receptor, rat testis, $\gamma 1$ subunit.*

INTRODUCTION

γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the vertebrate central nervous system (CNS)[1]. Whereas outside the CNS, many peripheral tissues have also been found to have GABAergic system[2].

The mammalian sperm acrosome reaction (AR) is a modified exocytotic event that is essential to the fertilization process[3]. Two main agonists of AR, the zona pellucida glycoprotein ZP3[4] and progesterone[5], have been identified in the oocyte vestments. Recent studies have suggested that progesterone appears to initiate the human AR by acting at a novel type of steroid receptor on the sperm plasma membrane but not by the classical nuclear receptor[6-10]. Meantime, a great deal of researches so far suggested the participation of a neuronal-like GABAA recepor/Cl^- channel in progesterone-initiated mammalian sperm AR[11],[12]. Moreover, it is also found that GABA at relatively low concentrations may mimic the effects of progesterone: increasing the fraction of acrosome-reacted cells in capacitated human and mouse spermatozoa[10],[13]. However, it is a conspicuous lack about the molecular characteristics of this receptor on mammalian spermatozoa. Given the promotion in understanding the functional mechanism of progesterone on mammalian spermatozoa, it is significant to identify the subunits composition of sperm $GABA_A$ receptor.

All natural $GABA_A$ receptors, found in vivo so far, containing α, β and γ subunits, meanwhile, unique pharmacological profiles could be conferred by

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GABAA receptors containing different g subunits [14]. In previous work (unpublished), we have found α5, β1, β2, and β3 subunits of GABAA receptor were expressed in rat testis and finally localized onto sperm head. Thus, we designed experiments to examine which type of γ subunits exists in rat testis. And the identification of expression type of γ subunits in testis might provide significant structural information for the understanding about the specific effects of $GABA_A$ receptors on sperm AR.

MATERIALS AND METHODS

Preparation of mRNA from rat testis

Rat (Wistar male, 90 days of age) testis was freshly excised. Testis total RNA was extracted using Trizol (Gibco-BRL) according to the procedure described by the manufacturer. mRNA was isolated using Oligotex Kit (Qiagen).

RT-PCR

Rat testis first strand cDNA was synthesized using Superscript Preamplification System (Gibco-BRL).

Two degenerate PCR primers were designed based on the conserved domain among three members of GABAA receptor g subunits as

primer 1:

5'- CACTGGATA(C)ACA(C,G)ACG(A,T)CCCA-3'

(corresponding to g1 cDNA sequence: 574-592);

primer 2:

5'-GCAG(C)GGA(G)ATG(A)TAG(C)GTCTGG(A)-3'

(corresponding to g1 cDNA sequence: 924-942).

Primers were designed to encompass the intronic sequences, so that any PCR product amplified from genomic DNA contaminating the RNA preparation could be distinguished. The PCR reaction was performed initially by denaturation at 95℃ for 4 min, and then 30 cycles at 94℃ for 1 min, 50℃ for 1 min, 72℃ for 1 min followed by extension for 8 min at 72℃. The primary PCR product was diluted and then used as template to perform a secondary reaction and the reaction condition is as same as the first round except for the annealing temperature increasing to 55℃.

PCR products were TA-cloned into PGEMT easy vector (Promega) and sequenced.

Northern blot analysis

The existence of γ1 mRNA was examined by performing Northern blotting. Isolated poly(A)$^+$ RNA (1-3 mg each lane) was electrophoresed in a 1% (w/v) formaldehyde agarose gel, capillary blotted onto a nylon membrane and UV cross-linked. Probes (25 ng each) were random labeled with [^{32}P]dCTP to a specific activity of > 1× 10^9 cpm. Blots were hybridized in a standard 50%(v/v) formamide buffer[15] at 42 ℃ and washed in 0.2 × SSC, 0.1 (w/v) %SDS at 55℃, before exposure to X-ray film with an intensifying screen. Autoradiographs were exposed for 24 h or longer.

In situ hybridization

In situ hybridization of rat testis cryostat sections was performed as Long A.A. described[16]. Sense and antisense riboprobes were prepared with T7/ SP6 DIG RNA Labeling Kit (Roche). Hybridization signal was detected by using DIG Nucleic Acid Detection Kit (Roche).

RESULTS

RT-PCR

As shown in Fig 1, after performing RT-PCR

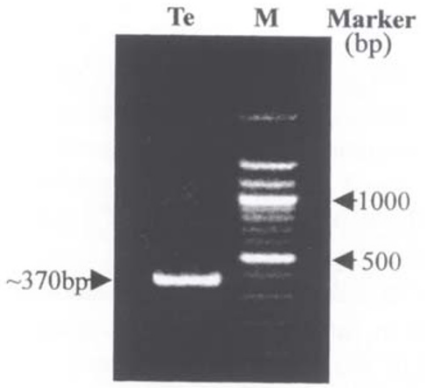

Fig 1. RT-PCR analysis of g1 subunit of GABAA receptor in rat testis. Testis mRNA was reverse-transcribed and PCR was performed using degenerate primers. The lengths of the products were ~370 bp. Te. testis; M. 100bp ladder marker.

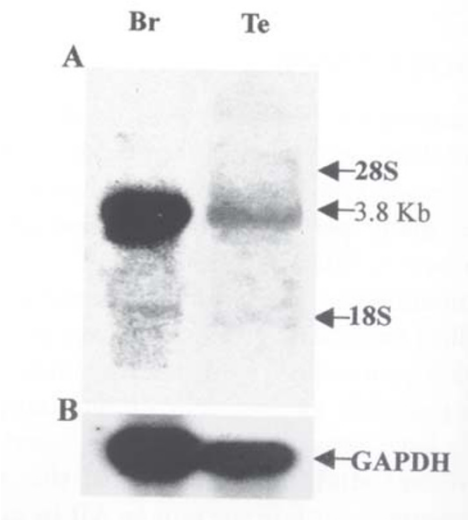

Fig 2. Northern Blot analysis of γ_1 subunit mRNA of GABAA receptor in rat testis. A, The blot was hybridized with cDNA fragment (obtained from RT-PCR) radiolabeled by random priming. A ~ 3.8 kb band can be detected both in brain and testis sample with much lower level in testis sample. Te, rat testis poly(A)$^+$RNA; Br, rat brain poly(A)$^+$ RNA; B, The same blot probed with radiolabeled house-keeping GAPDH cDNA.

with degenerate primers, an intense ~370 bp band which corresponding in size to the fragments of predicted brain γ subunits was obtained from testis mRNA specimen. Cloning and sequencing this fragment, results showed that only the transcript of $\gamma 1$ subunit fragment, but not that of $\gamma 2$ or $\gamma 3$ subunit fragment, exist in rat testis mRNA. The sequence of cloned $\gamma 1$ subunit fragment in testis is exact identical to that in brain (seq: 574-942).

Northern blot analysis

Using the fragment obtained from RT-PCR as probe and labeled by [^{32}P]dCTP, we performed Northern blot analysis. As shown in Fig 2A, rat testis mRNA contains one hybridizing bands at ~ 3.8 kb which is identical in size to the γ_1 band detected in rat brain mRNA. It is obvious that much lower level of γ_1 expression was found in testis compared with that in brain. Result of hybridizing to housekeeping gene GAPDH with same blot was shown in Fig 2B.

In situ hybridization

As shown in Fig 3A, C and D, results of in situ hybridization with DIG-labeled antisense γ_1 probe showed that γ_1 transcript localizes in round spermatid and spermatozoa, maybe also in secondary spermatocyte, region of rat testis section, but not in spermatogonia, Leydig cells and Sertoli cells; while with DIG-labeled sense γ_1 probe didn't show the positive signal, as shown in Fig 3B.

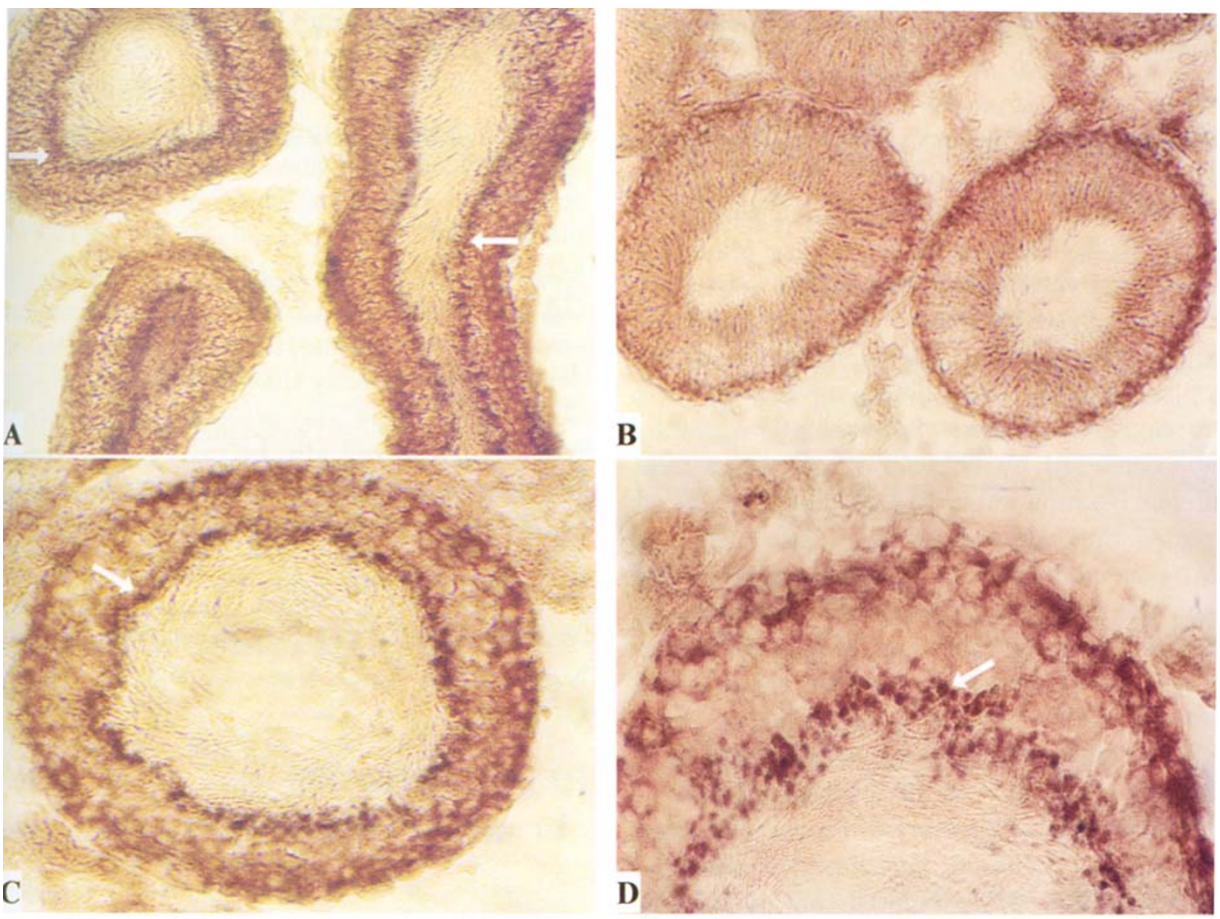

Fig 3. *In situ* hybridization analysis of GABAA receptor in rat testis cryostat sections. g1 subunit transcript was detected in testis cryostat sections with digoxigenin-labeled RNA probe. ? A (60×), C (120×) and D (240×): using antisense probe; B (60×): using sense probe as a control. Arrows indicate strong signals which are present in round spermatids and spermatozoa, maybe also in secondary spermatocyte region of seminiferous tubule sections (A, C and D), no signals are encountered in spermatogonia, Leydig cells and Sertoli cells. Sense probe doesn抉 detect positive signals (B).

DISCUSSION

Our evidences directly showed that the transcript of γ_1 subunit of $GABA_A$ receptor exists exclusively in rat testis. Based on the results of Northern blotting, it is obvious that the level of γ_1 subunit in rat testis is much lower than that in rat brain, and this is in consistent with the finding that g1 subunit band was obtained from testis cDNA after second PCR amplification. Meanwhile, results of in situ hybridization localized the expression of γ_1 subunit in round spermatids and spermatozoa, maybe also in secondary spermatocyte, but not in other cell types in rat testis.

Progesterone or GABA can induce mammalian sperm acrosome reaction in vitro; and previous studies have presumed that, after stimulated by progesterone or GABA, the depolarization of sperm membrane and calcium influx induced by the activation of GABAA receptors on spermatozoa can lead to the sperm acrosome reaction[11]. These "excitatory" effects induced by activation of $GABA_A$ receptors on spermatozoa opposite to the classical inhibitory effects of $GABA_A$ receptors in mature mammalian CNS [17]. However, it is not clear about the mechanism responsible for these effects yet. Electrophysiological studies showed that $GABA_A$ receptors with different subunits composition have different physiological function; moreover, evidences in vitro suggested unique pharmacological profiles could be conferred by $GABA_A$ receptors containing different gamma subunits[14]. For example, it is suggested that the positive modulatory effect of DMCM, one modulator of $GABA_A$ receptor, depends on the presence of g1 subunit in the native $GABA_A$ receptors[18]; moreover, studies on recombinant $GABA_A$ receptors showed that the presence of γ1 subunit induced higher neurosteroid modulatory efficacy on $GABA_A$ receptors compared with the presence of γ_2 subunit [19]. In contrast to γ_2 subunit being abundantly expressed in mature mammalian CNS[20],$\gamma_1$subunit is exclusively expressed in rat testis. Thus, we speculated that γ_1 subunit in rat testis might contribute to the specific function of $GABA_A$ receptors on the spermatozoa. Interestingly, other studies also suggested a possible relationship between γ_1 subunit and excitatory function of GABAA receptors in a few cases. It is accepted that during early development stage GABA exerts mainly an excitatory action via $GABA_A$ receptors through membrane depolarization and a rise in intracellular Ca^{2+}, and these effects disappeared during development[21], [22], while studies on distribution of subunits of $GABA_A$ receptor revealed that g1 subunit was strongly expressed during early development stage and with development its expression dropped markedly and restricted to relatively few structures[23], whereas γ_2 subunit becomes the mainly expression type of gamma subunit in mature CNS[20]. Additionally, the excitatory effects of $GABA_A$ receptors were also proved in bromodeoxyundine (BrdU)+ neural precursor cells and γ_1 is also the expression pattern of gamma subunit in these cells[24]. In conclusion, we hypothesized that γ1 subunit might be a molecular determinant of the excitatory function of $GABA_A$ receptor in rat spermatozoa.

Our evidences proved directly that γ_1 subunit is the only type of gamma subunits expressed in rat testis and thus confirmed the expression of $GABA_A$ receptor in rat testis. This study provided the significant structural information for making investigation into the "excitatory" function mechanism of $GABA_A$ receptors in mammalian fertilization process. Moreover, due to previous studies having demonstrated directly that $GABA_B$ receptor[25] and GAT-1[26],[27] are also present on spermatozoa, so further studies will also be carried out to examine the possible relationship between $GABA_A$ receptor, GABAB receptor and GAT-1 in sperm AR.

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