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Identification of glutamate transporters and receptors in mouse testis[1]

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ABSTRACT

AIM: To investigate the presence of glutamate transporters and receptors in mouse testis. **METHODS:** Glutamate uptake analysis was performed to study the function of glutamate transporters in mouse testis. Comparative RT-PCR technique and sequencing analysis were used to study the expression of glutamate receptors and transporters in mouse testis. **RESULTS:** Mouse testis possessed glutamate uptake capacity with sodium-dependence. V_{max} value of glutamate uptake was (1.60 ± 0.21) pmol/min per mg protein and K_m value of glutamate uptake was (11.0±1.6) μmol/L in mouse testis according to saturation analysis. Furthermore, the uptake activity could be inhibited by DHK (GLT1 selective inhibitor) and THA (glutamate uptake inhibitor). In addition, RT-PCR results revealed that glutamate transporters (GLT1 and EAAC1) and ionotropic glutamate receptors (NR1, NR2B, GluR6 and KA2) were expressed in mouse testis. **CONCLUSION:** Glutamate transporters and receptors do exist in mouse testis.

INTRODUCTION

Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS). Its neurotransmission can be mediated by various ligand-gated ion channels, of which there are three subtypes. These subtypes, which are classified on the basis of sequence homologies and agonist affinities, are *N*-methyl-*D*-aspartate (NMDA) receptors (NR1 and NR2A-D), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (GluR1-4), and kainate (KA) receptors (GluR5-7 and KA1&2)[1,2]. The termination of glutamate actions depends on its transporters by the mechanism of rapid reuptake. Members of the sodium-dependent glutamate transporter family identified thus far include GLAST, GLT1, EAAC1, EAAT4, and EAAT5[3]. All glutamate receptors and transporter subtypes are expressed in CNS, where they have been implicated in many aspects of physiology and pathology[4-6]. How-ever, the presence and the role of glutamate receptors and transporters in the peripheral tissues have remained unclear. It has been shown that some of glutamate receptors exist in retina, adrenal, pituitary, pineal, and pancreatic islets[7,8], and some of glutamate transporters are present in liver, kidney, intestine, heart, lung,

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skeletal muscle, bone marrow, and placenta[3,6]. Recent research showed that metabotropic glutamate receptors were expressed in the rat and human testis[9].

Although glutamate concentration and total content were determined in the post-natal rat testis by an enzymatic method as early as 1970[10], little is known about its role. A well-studied neurotransmitter in male reproductive system is γ-aminobutyric acid (GABA), the principle inhibitory neurotransmitter in the mammalian CNS. The concentration of GABA was determined in epididymis, seminal vesicle, and testicle of the adult rat[11], and a direct effect on steroidogenesis and sperm viability and motility has been described[12]. Recently it is shown that $GABA_A$ receptor subunits are expressed in multiple rat endocrine tissues including adrenal, ovary, testis, placenta, and uterus in a tissue specific manner[13]. The existence of $GABA_B$ receptor and GABA transporter subtype I (GAT1) was identified in mouse and rat testis and sperm[14-16]. GABA uptake was detected in mouse testis and transgenic mice overexpressing GAT1 showed reduced mass and size of testis as compared with wild-type mice[16]. These findings suggest that GABA may regulate sperm functions such as capacitation and acrosome reaction via its interaction with the receptors and transporters that were originally found in CNS[17-19]. Because brain GABA system exists in testis and has functions described as above, it is reasonable to infer that brain glutamate system is present in testis and may have functions other than neurotransmission similar to GABA in some way. In the present study, we found that testis possessed glutamate uptake property in similar way as brain did and identified the presence of glutamate transporters and receptors in mouse testis using RT-PCR techniques.

MATERIALS AND METHODS

Materials All chemicals were analytical grade unless otherwise stated. C57BL/6J mice were group housed in plastic mouse cages with free access to standard rodent chow and water. The colony room was maintained at 22 ºC±2 ºC with a 12 h:12 h light:dark cycle. All the experimental protocols were performed in compliance with the National Institutes of Health Guidelines for the care and use of animals and were approved by the local Animal Care and Use Committee.

Analysis of [^{3}H]-glutamate uptake in testis Testis and liver samples were removed from adult C57BL/6J male mice. The testicular and liver cell suspensions were prepared using mechanical dissection methods in aCSF buffer (containing in mmol/L: NaCl 126; $NaHCO_3$ 27.4; KCl 2.4; KH_2PO_4 0.49; $CaCl_2$ 1.2; $MgCl_2$ 0.83; Na_2HPO_4 0.49; *D*-glucose 7.1; pH 7.2-7.4) or modified aCSF buffer in which NaCl was partially substituted by LiCl with NaCl 60 mmol/L final concentration and fully substituted by LiCl served as control. The same aliquot of testicular and liver cell suspensions was lysised with NaOH 2 mol/L to quantify the protein concentration using BCA reagent (Pierce). Testicular cells were pre-incubated in aCSF buffer or modified aCSF buffer gassed with 95 % O_2/5 % CO_2 for 5 min at 37 ºC. Then uptake was initiated by the addition of a mixture of cold and tritiated glutamate (glutamate, Sigma; [^{3}H]-glutamate, Amersham Pharmacia Biotech).

For time-course studies, the final concentration of the compound was 100 nmol/L, 10 % of which was tritiated. For saturation analysis, the amount of [^{3}H]-glutamate used was kept constant and the different glutamate concentrations (0.1-40 μmol/L) were obtained by adding varying amount of unlabelled compounds. After 20 min incubation, uptake was terminated by vacuum filtration through Whatman glass-fiber filters. The glutamate content of the filters was assayed by liquid scintillation counter (Beckman), taking dilution factors into account. For kinetic data analysis, linear fit was done to calculate V_{max} and K_m values according to Michaclis-Menten equation ($V=V_{max}S/K_m+S$).

Dihydrokainic acid (DHK) and *DL*-thero-β-hydroxyaspartic acid (THA) were dissolved in water and mixed with glutamate and [^{3}H]-glutamate in appropriate concentration. Synaptosomes were prepared as we described previously[20]. Uptake experiments were performed as described above.

Comparative RT-PCR analysis Adult C57BL/6J male mice were anesthetized with sodium pento-barbital, and consequently perfused intracardially with phosphate-buffered saline (PBS). After perfusion, brain and testis samples were removed immediately. Total RNA was extracted with Trizol reagent (Gibco BRL) as detailed by the manufacturer. RNA integrity was identified by formaldehyde-electrophoresis. RNA sample was thoroughly treated with RNase-Free DNase (5 U/μg RNA) for 45 min at 37 ºC before reverse-transcription performed with Gibco kit.

Primers of glutamate transporters and receptors were synthesized according to respectively selected sequences. These oligonucleotide primers and PCR conditions were given in Tab 1. Meanwhile, GAPDH mRNA was detected by PCR as an internal standard

Tab 1. Selected PCR primers for glutamate receptor and transporter subunit mRNA5.

Product	Product size (bp)	Primer (5' to 3')	Position (GeneBank No)
mGLT1	667	AGAGGCTGCCCGTTAAATACCG GTAATACACCATAGCTCTCGC	-101-566 (AB007810)
mEAAC1	564	ACCGGAATCACTGGCTGCTGCTCTC CCCACGATCTTGTATTCCTTTGTC	45-608 (D43797)
mNR1	512	GTAAACCAGGCCAATAAGCGACACG GCTTCCAGGTCCCGGCTTCCATC	135-646 (NM_008169)
mNR2B	693	ACAGACACGGTGCTTCAGAGTTCC GCCGTTCCAGGTCCCGTTGATTTTC	796-1488 (NM_008171)
mGLUR6	490	GCTGCCATCTTCGGTCCTTCAAC CCACATCAAGAGCGAAGAGGTCC	310-799 (D10054)
MKA2	698	AGCGGGACAGCCAGTACGAGAC GGACAGCGCAGGGCCAGGATAG	212-909 (D10011)

with primers: 5'-ACGACCCCTTCATTGACC-3' (forward) and 5'-CCAGTGAGCTTCCCGTTCAGC-3' (reverse), which spanned 588 bp nucleotides within the coding sequence for GAPDH. Resultant GAPDH RT-PCR products were referred to quantify the expression level of target products. RT-omitted RNA samples were directly amplified by PCR with 5-fold amounts of the same aliquot to demonstrate the amplified products were mRNA-based instead of genomic DNA-based.

The PCR was performed initially by denaturing template DNA at 95 ºC for 5 min, followed by minimized cycles of 94 ºC for 45 s, 60 ºC for 45 s and 72 ºC for 1 min, and then followed by a final extension at 72 ºC for 10 min. The annealing temperatures for different primer pairs were altered in the range of 64 ºC to 58 ºC depending upon T_m (melting temperature) of the primer pairs in use. Amplified DNA fragments were separated by agarose gel (1.2 %), purified and sequenced for confirmation. Relative intensities of the products were estimated with Molecular Imager FX (BIO-RAD).

Statistical analysis Results were expressed as mean±SD. The data were analyzed by one-way analysis of variance (ANOVA). $P<0.05$ were regarded as statistically significant.

RESULTS

Analysis of [^{3}H]-glutamate uptake in testis [^{3}H]-glutamate uptake test was conducted in mouse testis. Results showed that mouse testis possessed glutamate uptake activity (Fig 1A-C). The time-course analysis indicated that the testicular accumulation of glutamate was time-dependent and sodium-dependent, which are the essential characteristics possessed by glutamate transporters (Fig 1A). V_{max} value [(1.60 ± 0.21) pmol/min per mg protein] and K_m value [(11.0 ± 1.6) μmol/L] was calculated according to saturation analysis (Fig 1B, C).

Furthermore, we determined whether glutamate uptake could be blocked by DHK (GLT1 selective inhibitor) and THA (glutamate uptake inhibitor). Glutamate uptake in synaptosomes from mouse brain showed that THA (100 μmol/L) could significantly block glutamate uptake while DHK (100 μmol/L) just slightly reduced glutamate uptake (Fig 2A), because GLT1 is mainly expressed in glial cells and less in synaptosomes[21], which serve as a positive control. In testis, glutamate uptake was significantly inhibited by DHK and THA (Fig 2B). This result indicated that GLT1 contributing to the glutamate uptake activity. The inhibitory effect of THA on glutamate uptake was more potent than that of DHK, which suggested that there were other glutamate transporters in testis besides GLT1. In addition, there was almost no glutamate uptake in liver, which served as a negative control (Fig 2B).

Comparative RT-PCR and sequence analysis The expression and expression level of two glutamate transporters (GLT1, EAAC1) and four glutamate receptors (NR1, NR2B, GluR6, and KA2) in mouse testis were assessed by comparative RT-PCR analysis (Fig 3A-D). Specific fragments from mouse testis RT samples were obtained using selective primers and products of corresponding size from mouse brain RT samples via PCR with the same primers were also obtained. Subsequently, the amplified products were confirmed

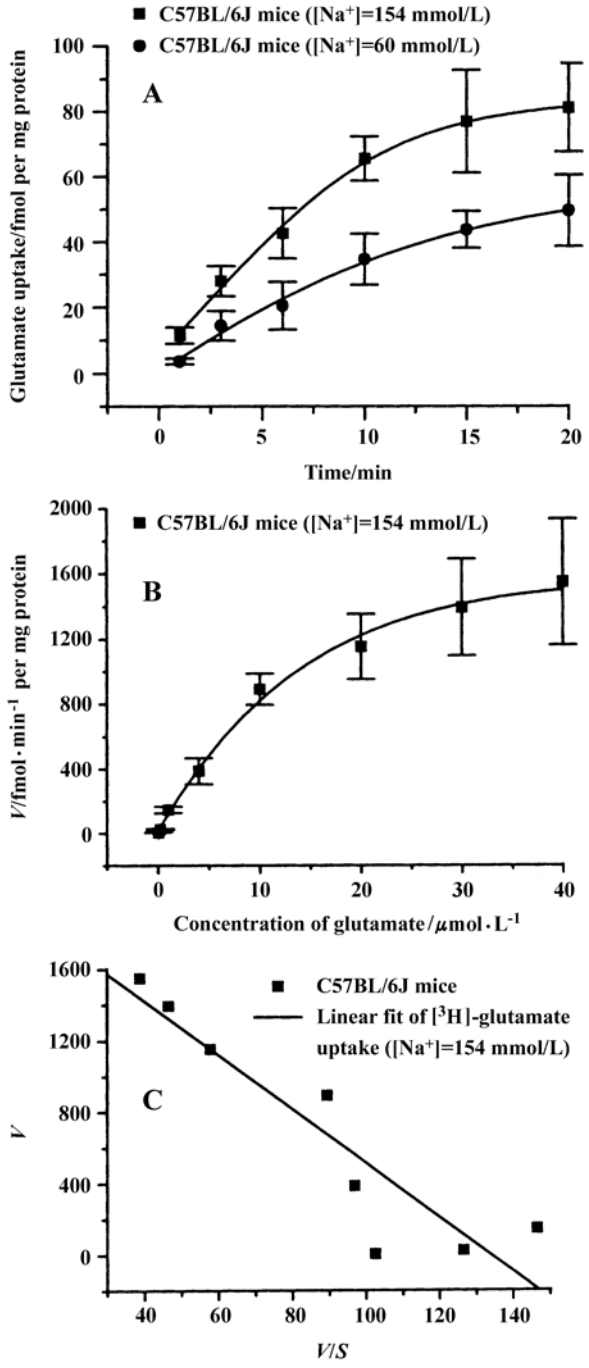

Fig 1. [^{3}H]-glutamate uptake in mouse testis. (A) Time-courses of [^{3}H]-glutamate uptake in testis, showing that glutamate uptake process in testis is sodium-dependent. (B), (C) Saturable uptake of glutamate in testis. (B) *V vs S* plot. (C) *V vs V/S* plot. *n*=9 in three independent experiments. Mean ± SD.

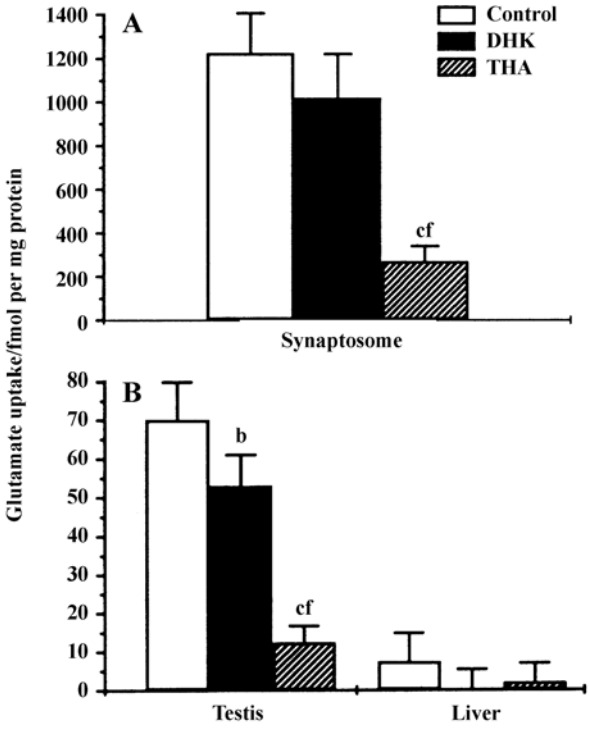

Fig 2. [^{3}H]-glutamate uptake in mouse testis in the presence of glutamate inhibitors. (A) Glutamate uptake of synaptosomes from mouse brain in three minutes incubation at 37 °C when using glutamate uptake inhibitors (100 μmol/L). DHK: Dihydrokainic acid; THA: *DL*-thero-β-hydroxyaspartic acid. (B) Glutamate uptake of mouse testicular cells (10 min incubation) and mouse liver cells (10 min incubation) when using glutamate uptake inhibitors (100 μmol/L). DHK: dihydrokainic acid; THA: *DL*-thero-β-hydroxyaspartic acid. *n*=8 in two independent experiments. Mean±SD. ^{b}*P*<0.05, ^{c}*P*<0.01 *vs* control; ^{f}*P*<0.01 *vs* DHK.

by sequencing analysis. It was found that the products from mouse testis were identical to those from mouse brain. Furthermore, different expression levels of these genes in testis were revealed compared with those in brain when normalized to GAPDH mRNA level (Fig 3E).

It is noteworthy that the expression level of KA2 in testis is almost as high as that in brain. No specific PCR product from the RNA samples that were omitted reverse transcription could be observed over background, which verified the absence of genomic DNA contamination (Fig 3D).

DISCUSSION

GABA system existing in testis was reported by several laboratories[13-16] and important function of GABA in male reproductive system was demonstrated[12]. In the present study, we provided the first evidence that glutamate system including glutamate transporters (GLT1 and EAAC1) and receptors (NR1, NR2B, GluR6, and KA2) also existed in mouse testis. As we know, the central nervous system (CNS) and the male reproductive system possess blood barriers (blood-brain bar-

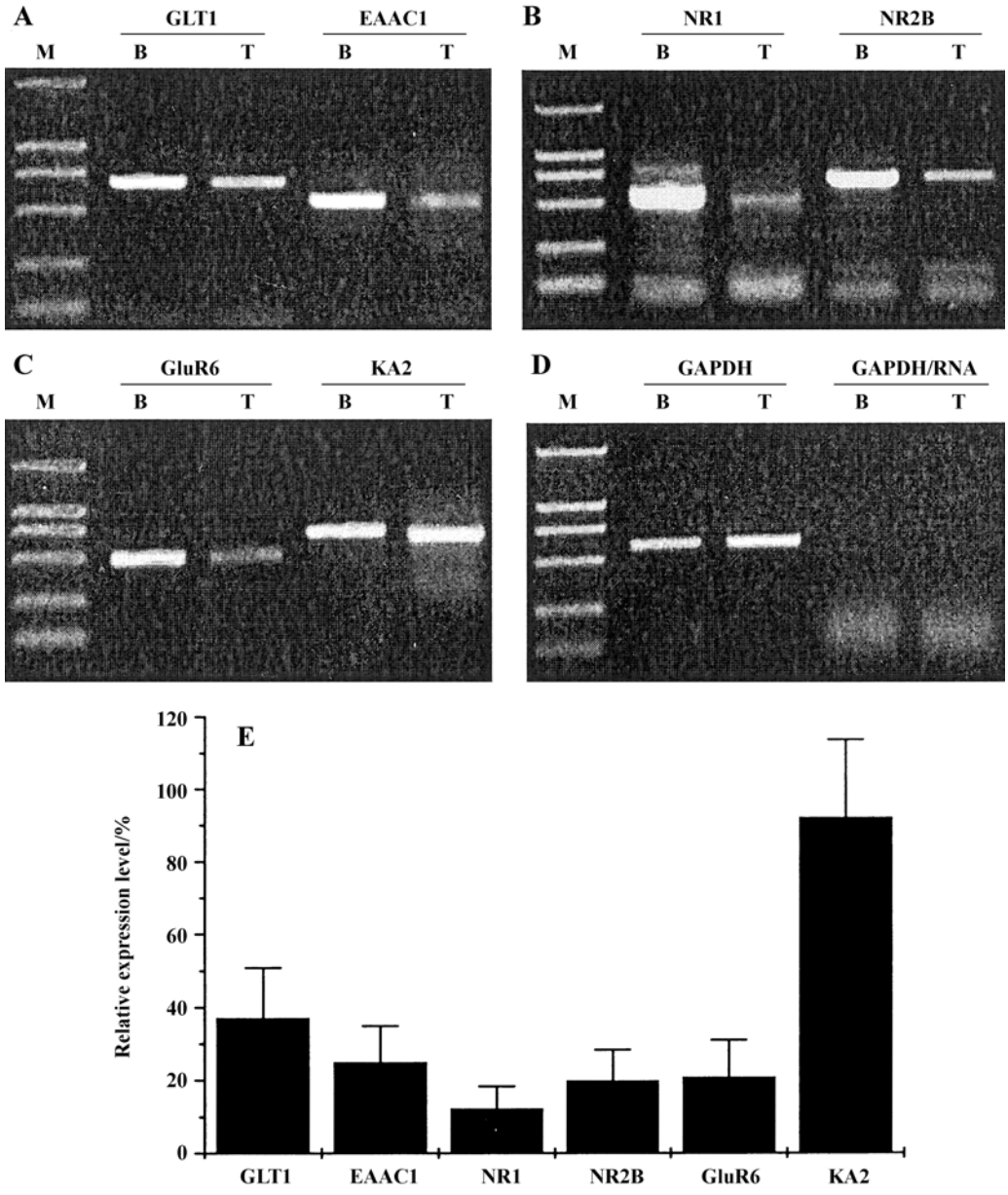

Fig 3. Comparative RT-PCR analysis of the expression of glutamate receptors and transporters in mouse brain (B) and mouse testis (T). M: DNA marker (2000, 1000, 750, 500, 250, and 100 bp). (A), (B), (C) Indicating the amplification of cDNA of glutamate transporters (GLT1, EAAC1) and receptors (NR1, NR2B, GluR6, and KA2) with RT-conducted RNA samples using selective primers. (D) Indicating the amplification of GAPDH cDNA served as an internal standard and the direct amplification with RT-omitted RNA samples using GAPDH primers. (E) Revealing relative expression levels about the glutamate receptors and transporters in testis compared with those in brain. *n*=3. Mean±SD.

rier and blood-testis barrier) and need adequate concentration of blood glucose. This phenomenon is noteworthy and interesting, but up to now its mechanism is less clear. Our findings made the issue of similarity in gene expression pattern between CNS and the male reproductive system more intriguing.

The time-course analysis demonstrated that glutamate uptake capacity was sodium-dependent, which is one of the essential characteristics possessed by glutamate transporters. K_m value determined by kinetic analysis was consistent with reported range (1-100 μmol/L) despite the weak uptake capacity in testis compared with that in brain (Fig 2A)[22]. Furthermore, glutamate uptake detected in mouse testis could be blocked by glutamate uptake inhibitors, and THA (glutamate uptake inhibitor) showed more potent than DHK (GLT1

selective inhibitor) (Fig 2B), suggesting that there were other glutamate transporters in testis besides GLT1. This result lent another support to the existence of EAAC1 (Fig 3A). Further studies should be conducted to reveal the location of these proteins in testis.

Existence of glutamate in testis may act as the precursor of GABA since glutamic acid decarboxylase (GAD), the key enzyme in the synthesis of GABA, is shown to express in the testis of several different species[23]. But the appearance of glutamate transporters and receptors in testis implied that glutamate existing in testis must have other physiological functions. It was validated that GABA can mimic and potentiate the action of progesterone in initiating the acrosome reaction (AR) of mammalian sperm[17,24]. It is also assessed the influence of increased amount of GAT1 on the testicular morphology with a transgenic model[16]. We suppose that glutamate system possesses physiological functions and can be therapeutically targeted for contraception or dysgenesis treatment.

Taken together, we demonstrated that brain glutamate system was present in mouse testis. All findings mentioned lent strong support to implication that glutamate might have unknown effects in testis other than as the precursor of GABA or as neurotransmitter in brain.

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