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Identification of Glutamate Receptors and Transporters in Mouse and Human Sperm

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ABSTRACT: γ-Aminobutyric acid (GABA) and glutamate (Glu) are considered as the predominant inhibitory and excitatory neurotransmitters in mammalian central nervous systems (CNS), respectively. The presence of the GABA system and metabotropic glutamate receptors in sperm prompted us to explore the existence of ionotropic glutamate receptors and glutamate transporters in sperm. Immunofluorescent analysis was used to investigate the existence and location of glutamate, glutamate receptor (NR2B), and glutamate transporter (GLT1) in mouse and human sperm. Our present results showed that NR2B was located in the midpiece of sperm, whereas GLT1 mainly existed in the head. Moreover, glutamate uptake activity was detected in mouse sperm and it could be blocked by dihydrokainic acid (DHK, GLT1-selective inhibitor) and DL-threo-beta-hydroxyaspartic acid (THA, nonselective inhibitor). In addition, reverse transcription-polymerase chain reaction technique and sequencing analysis revealed that glutamate transporters (GLT1 and EAAC1) and ionotropic glutamate receptors (NR1, NR2B, GluR6, and KA2) existed in mouse sperm as well as in human sperm. The present findings are the first direct evidence for the existence of ionotropic glutamate receptors and glutamate transporters in sperm. It also indicates that, in sperm, glutamate receptors and transporters might have functions other than neurotransmission.

Key words: Uptake, immunocytochemistry, RT-PCR, inhibitor.
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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 the N-methyl-D-aspartate (NMDA) receptors (NR1 and NR2A–D), the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (GluR1–4), and kainate (KA) receptors (GluR5–7 and KA1&2) (Nakanishi, 1992; Hollmann and Heinemann, 1994). 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 (Kanai and Hediger, 1992; Pines et al, 1992; Storck et al, 1992; Fairman et al, 1995; Arriza et al, 1997). All glutamate receptor and transporter subtypes are expressed in CNS, where they have been implicated in many physiological and pathological aspects (Collingridge and Lester, 1989; Sims and Robinson, 1999). However, the presence of glutamate receptors and transporters and their functions in the peripheral tissues still remain unclear. It has been shown that some glutamate receptors exist in retina, adrenal, pituitary, pineal, and pancreatic islets (Inagaki et al, 1995; Hinoi and Yoneda, 2001) and some glutamate transporters are present in liver, kidney, intestine, heart, lung, skeletal muscle, bone marrow, and placenta (Robinson, 1999; Sims and Robinson, 1999). Recent research showed that metabotropic glutamate receptors were expressed in the rat and human testis (Storto et al, 2001).

Although glutamate concentration and total content were determined in the postnatal rat testis by an enzymatic method as early as 1970 (Harkonen et al, 1970), little was known about its function. A well-studied neurotransmitter in male reproductive system is γ-aminobutyric acid (GABA), the principle inhibitory neurotransmitter in mammalian CNS. The concentration of GABA was determined in epididymis, seminal vesicle, and testicle of the adult rat (Erdo et al, 1983) and a direct effect on steroidogenesis and sperm viability and motility has been described (Frungier et al, 1996). Recently, it has

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Selected PCR primers for glutamate receptor and transporter subunit mRNAs

Product	Product Size (bp)	Primer (5′ to 3′)	Position (GeneBank No.)
mGLT1 (transporter)	667	AGAGGCTGCCCGTTAAATACCG GTAATACACCATAGCTCTCGC	−101–566 (AB007810)
mEAAC1 (transporter)	564	ACCGGAATCACTGGCTGCTGCTCTC CCCACGATCTTGTATTCCTTTGTC	45–608 (D43797)
mNR1 (receptor)	512	GTAAACCAGGCCAATAAGCGACACG GCTTCCAGGTCCCGGCTTCCATC	135–646 (NM_008169)
mNR2B (receptor)	693	ACAGACACGGTGCTTCAGAGTTCC GCCGTTCCAGGTCCCGTTGATTTTC	796–1488 (NM_008171)
mGuR6 (receptor)	490	GCTGCCATCTTCGGTCCTTCAAC CCACATCAAGAGCGAAGAGGTCC	310–799 (D 10054)
mKA2 (receptor)	698	AGCGGGACAGCCAGTACGAGAC GGACAGCGCAGGGCCAGGATAG	212–909 (D10011)
hGLT1 (transporter)	489	GGGCTTCTTCGCTTGGCATCTC CTCCGGCACCTCAGTCACAGTC	184–672 (U03505)
hEAAC1 (transporter)	496	TTCTAATGCGGATGCTGAAACTC CCAAAGACAAGGCAAAAGACAATC	176–6761 (HSU03506)
hNR1 (receptor)	501	GCACAAGCCCAACGCCATCCAG CGGTATACAGTGGCAGCATCGTC	198–698 (L05666)
hNR2B (receptor)	461	AGCCCCATCATTCTTCTTTACTG TTCCCCACCCTTTCCCACTTCC	673–1133 (NM_000834)
hEAA4(GluR6) (receptor)	490	the same as mGluR6	310–799 (HSU16126)
hEAA2(KA2) (receptor)	631	AGCGGGACAGCCAGTACGAGAC ACAAACTCAGGGTAGAAGGGGTG	212–842 (S40369)

been 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 (Akinci and Schofield, 1999). The existence of $GABA_B$ receptor and GABA transporter subtype I (GAT1) was demonstrated in mouse and rat testis and sperm (Hu et al, 2000; Ma et al, 2000; He et al, 2001). Transgenic mice overexpressing GAT1 showed reduced mass and size of testis and impaired production of sperm as compared with wild-type mice (Ma et al, 2000, unpublished data). It was suggested that GABA might regulate sperm functions such as capacitation and acrosome reaction via its interaction with the receptors and transporters that were originally found in CNS (De las heras et al, 1997; Shi et al, 1997). We postulate that glutamate receptors and transporters are present in sperm and have functions other than neurotransmission, similar to GABA. In the present study, we demonstrated the presence of glutamate transporters and receptors in mouse sperm as well as in human sperm and then found that mouse sperm possessed glutamate uptake property in similar way as brain did.

Materials and Methods

Materials

All chemicals were analytical grade unless otherwise stated. Human semen samples were generously supplied by Tongji Hospital, Shanghai, China. They were collected by masturbation from men (age 21–43 years) after more than 3 days abstinence and were processed immediately after liquefaction. Anti-glutamate primary antibody was purchased from Sigma (St Louis, Mo) and anti-NR2B and anti-GLT1 primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Fluorescein isothiocyanate (FITC)-labeled secondary antibodies were purchased from Sigma. C57BL/6J mice were group housed in plastic mouse cages with free access to standard rodent chow (Shuzhou Laboratorial Animal Food Company, Shuzhou, China) and water. The colony room was maintained at 22 ± 2°C with a 12 hour:12 hour 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.

Sperm Slides Preparation

Mouse sperm samples were collected from the caudal epididymis of adult C57BL/6J mice and fixed in 4% paraformaldehyde in PBS for 30 minutes, smeared on gelatin-coated slides, dried, and fixed. Human semen samples were collected by masturbation from men (age 21–43 years) after more than 3 days abstinence. The semen was allowed to liquefy at 37°C for 30 minutes, and thereafter normozoospermia was verified (WHO, 1999). Sperm slides were done as in the method for the mouse sperm.

Immunofluorescent Analysis of Mouse and Human Sperm

Mouse and human sperm slides were rinsed in 0.01 M phosphate-buffered saline (PBS) and blocked with 10% goat or rabbit

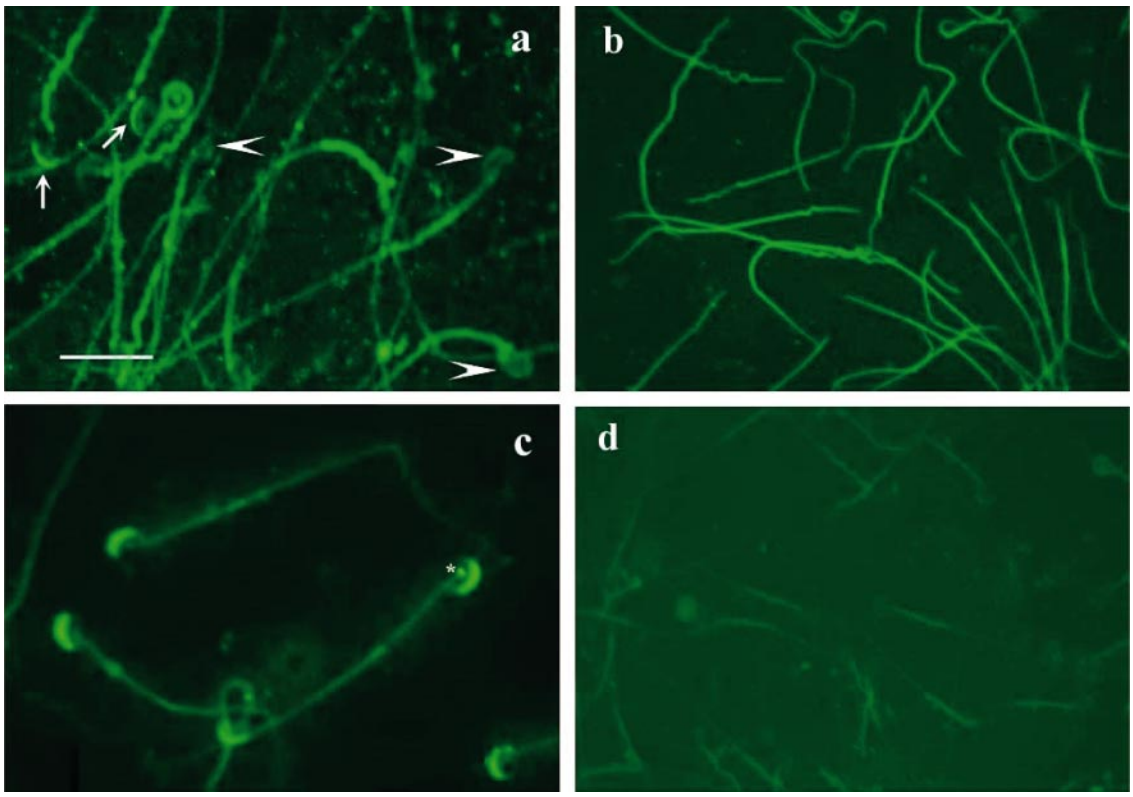

Figure 1. Immunofluorescent detection showing glutamate, NR2B, and GLT1 localized in mouse sperm. Scale bar represents 20 μm. **(a)** Note glutamate located in entire spermatid including head (arrows) while partially on tail (arrowheads). **(b)** Note NR2B was specifically located in the tail of sperm. **(c)** Note GLT1 was highly located in the head of the sperm with a weak signal in the sperm tail. * Points to nonspecific fluorescence staining. **(d)** Negative control of **(a), (b), (c)**.

serum plus 0.1% Triton X-100 in PBS to reduce nonspecific immunostaining and incubated with primary antibody or normal nonimmune serum as a negative control at 4°C overnight. After incubation, the slides were rinsed in PBS 3 times and exposed to corresponding secondary antibody for 2 hours at room temperature and then rinsed in PBS. Finally, slides were examined and photographed under a Leica fluorescence microscope.

Analysis of ^{3}H-glutamate Uptake in Mouse Sperm

Mouse sperm samples were collected from the caudal epididymis of adult C57BL/6J male mice after cervical dislocation. The caudal epididymis was cut into a few pieces in a small dish in Biggers-Whitten-Whittingham (BWW) buffer (containing, in g/L, NaCl, 5.54; KCl, 0.356; $CaCl_2 \bullet 2H_2O$, 0.250; KH_2PO_4, 0.162; $MgSO_4 \bullet 7H_2O$, 0.294; $NaHCO_3$, 2.1; glucose, 1.0; sodium pyruvic acid, 0.03; bovine serum albumin (BSA), 3.5; and 60% syrup of sodium lactic acid 3.7 mL/L) at 37°C. The sperm swum out from the caudal epididymis in a 1-hour incubation and were collected after gentle centrifugation. The percentage of sperm is no less than 95% in such a sperm suspension. Sperm were washed in aCSF buffer (containing in mM, 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). The sperm suspension was lysed with 2 mol/L NaOH to quantify the protein concentration using bicinchoninic acid (BCA) reagent (Pierce, Rockford). Sperm cells were preincubated in aCSF buffer gassed with 95% O_2/5% CO_2 for 5 minutes at 37°C. Then uptake was initiated by the addition of a mixture of cold and tritiated glutamate (glutamate, Sigma; ^{3}H-glutamate, Amersham Pharmacia Biotech, Piscataway, NY). The final concentration of the compound was 100 nM, 10% of which was tritiated. Samples incubated in a modified aCSF buffer, in which LiCl substituted NaCl, served as a background control. After 3 minutes of 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, Fullerton), taking dilution factors into account.

Dihydrokainic acid (DHK) and DL-threo-beta-hydroxyaspartic acid (THA) were dissolved in water and mixed with glutamate and ^{3}H-glutamate in appropriate concentrations. Mouse liver cells were isolated using mechanical dissection methods and prepared in aCSF buffer. Synaptosomes were prepared as described previously (Ma et al, 2001). Uptake experiments were performed as described above.

Reverse Transcription-Polymerase Chain Reaction Analysis

Mouse brain samples were removed from adult C57BL/6J male mice after cervical dislocation. Sperm samples were collected as described above. Total RNA was extracted with Trizol reagent (GIBCO BRL, Carlsbad, Calif) as detailed by the manufacturer. RNA integrity was identified by formaldehyde-electrophoresis.

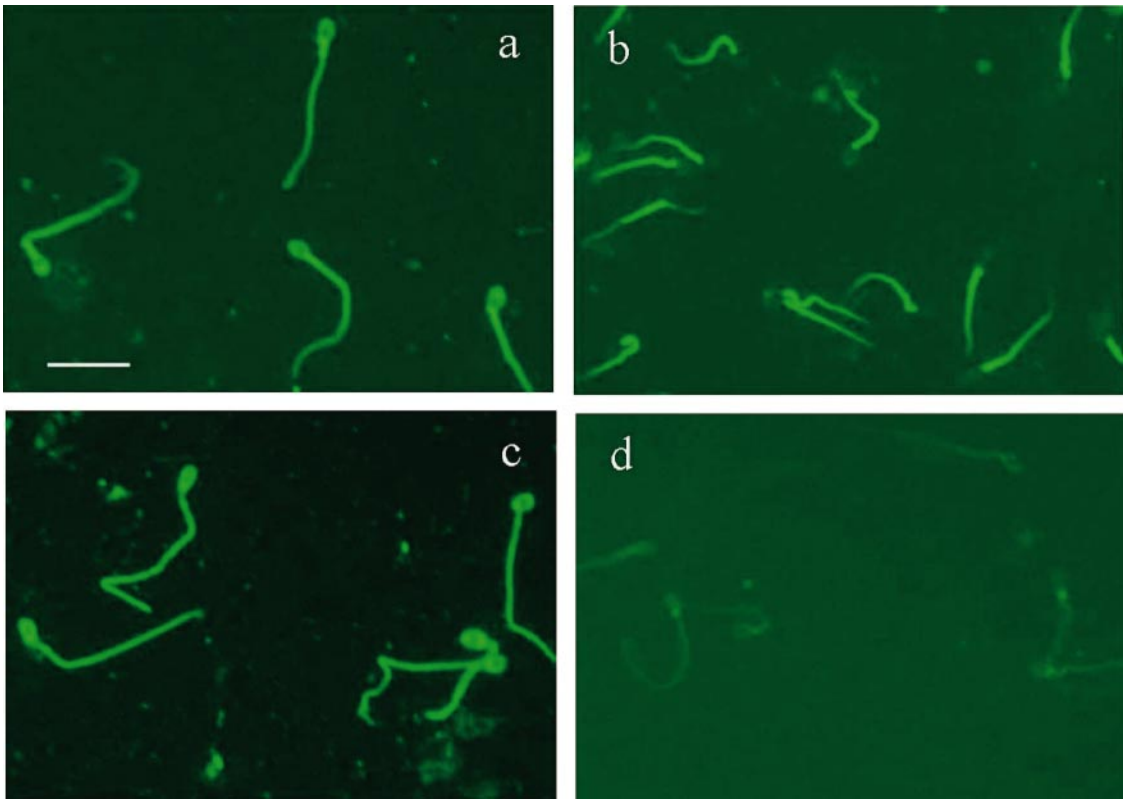

Figure 2. Immunofluorescent detection showing glutamate, NR2B, and GLT1 localized in human sperm. Scale bar represents 20 μm. **(a)** Note glutamate located in entire human sperm. **(b)** Note NR2B was specifically located in the middle piece of human sperm. **(c)** Note GLT1 was located in the head of the sperm as well as in the sperm tail. **(d)** Negative control of **(a), (b), (c)**.

RNA sample was thoroughly treated with RNase-Free DNase (5 U/μg RNA) for 45 minutes at 37°C before reverse-transcription (RT) performed with a GIBCO kit.

Primers of glutamate transporters and receptors were synthesized according to selected sequences. These oligonucleotide primers and polymerase chain reaction (PCR) conditions are given in the Table. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected by PCR as an internal standard 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 fivefold 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°C for 5 minutes, followed by minimized cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The annealing temperatures for different primer pairs were altered in the range of 64° to 58°C, depending on the 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, Hercules, Calif).

Human semen samples were collected by masturbation from men (age 21–43 years) after more than 3 days abstinence. The semen was allowed to liquefy at 37°C for 30 minutes. After liquefaction, a sample was immediately processed for RT-PCR. The RT-PCR was conducted as above. Human glyceraldehyde-3-phosphate dehydrogenase testis-specific (GAPDS) mRNA was detected by PCR as an internal standard with primers 5′-AGTAGAGCCCCAGCCACAACCAG-3′ (forward) and 5′-GAGGGCGCGGAGATGACCACAC-3′ (reverse), which spanned 483-bp nucleotides within the coding sequence for GAPDS.

Results

Immunofluorescent Analysis of Glutamate, NR2B, and GLT1 in Mouse Sperm

Employing immunofluorescent staining techniques, we found that glutamate was located in mouse sperm. It is worthy to note that, in some instances, glutamate was not present in heads of sperm but only at the tails (Figure 1a). For NR2B, it was clearly observed that the specific fluorescence was present in the midpiece of mouse sperm with no immunoactivity in the sperm head (Figure 1b). For GLT1, the intense immunofluorescence was in the head of the mouse sperm, with a weak signal in the sperm tail, which suggested that GLT1 was mainly expressed in

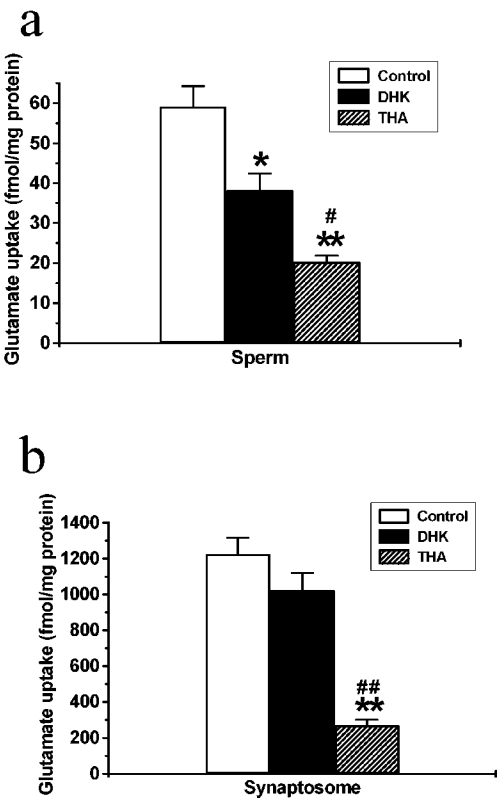

Figure 3. ^{3}H-glutamate uptake analysis in mouse sperm. **(a)** Glutamate uptake in mouse sperm using glutamate uptake inhibitors (100 μM). **(b)** Glutamate uptake of synaptosomes from mouse brain using glutamate uptake inhibitors (100 μM). DHK indicates dihydrokainic acid; THA, DL-threo-beta-hydroxyaspartic acid. Values are presented as mean ± SEM. * indicates $P < .05$; **, $P < .01$ versus control; #, $P < .05$, ##, $P < .01$ versus DHK (1-way ANOVA); n = 8 in 2 independent experiments.

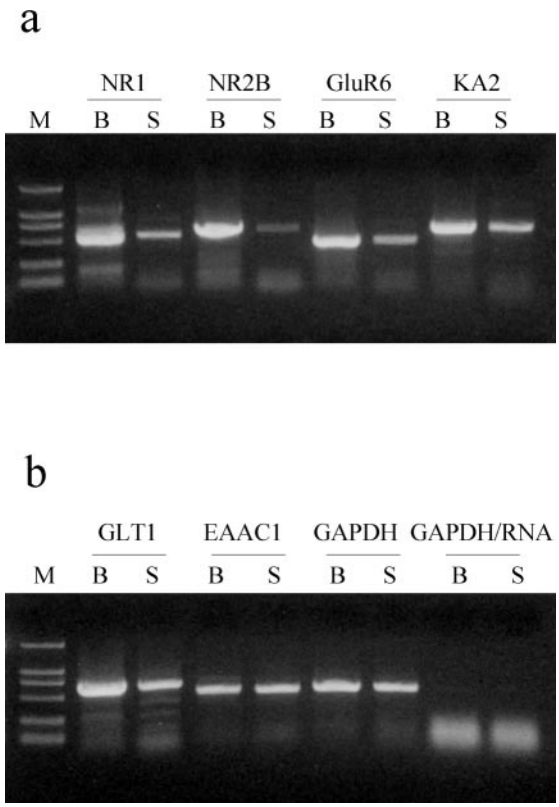

Figure 4. RT-PCR analysis of the expression of glutamate receptors and transporters in mouse brain (B) and mouse sperm (S). M: DNA Marker (2000, 1000, 750, 500, 250, 100bp). **(a)** Indicates the amplification of cDNA of glutamate receptors (NR1, NR2B, GluR6, KA2) with RT-conducted RNA samples using selective primers. **(b)** Indicates the amplification of glutamate transporters (GLT1, EAAC1) with RT-conducted RNA samples using selective primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA served as an internal standard and the direct amplification with RT-omitted RNA samples was conducted using GAPDH primers.

mouse sperm heads (Figure 1c). Figure 1d was the primary antibody-omitted negative control.

Immunofluorescent Analysis of Glutamate, NR2B, and GLT1 in Human Sperm

Glutamate was also found to be located on human sperm heads and tails (Figure 2a). For NR2B, it was clearly observed that the specific fluorescence was present in the midpiece of human sperm, with no immunoactivity in the sperm head (Figure 2b). For GLT1, the intense immunofluorescence was in the head of human sperm as well as in the sperm tail (Figure 2c). Figure 2d was the primary antibody-omitted negative control.

Analysis of ^{3}H-Glutamate Uptake in Mouse Sperm

To explore whether glutamate transporters possessed glutamate uptake activity, the ^{3}H-glutamate uptake test was conducted on mouse sperm. Results showed that mouse sperm possessed glutamate uptake activity (Figure 3a). In order to specify the glutamate uptake, we performed glutamate uptake experiments in the presence of DHK (GLT1-selective inhibitor) and THA (nonselective inhibitor). Glutamate uptake in mouse sperm was significantly inhibited by DHK (100 μM) and THA (100 μM) (Figure 3a). This result indicated that GLT1, the most important glutamate transporter in brain, contributed to the glutamate uptake activity. Because the glutamate uptake inhibitory effect of THA was better than that of DHK (Figure 3a), it suggested that there existed other glutamate transporters in sperm in addition to GLT1. Glutamate uptake in synaptosomal fraction from mouse brain showed that THA (100 μM) could significantly block glutamate uptake while DHK (100 μM) just slightly reduced glutamate uptake (Figure 3b) because GLT1 is mainly expressed in glial cells and less in synaptosomes (Rothstein et al, 1994). The data served as a positive control of glutamate uptake inhibitors (Figure 3b).

RT-PCR and Sequence Analysis in Mouse Sperm

The expression of four glutamate receptors (NR1, NR2B, GluR6, KA2) and two glutamate transporters (GLT1, EAAC1) in mouse sperm were assessed by RT-PCR anal-

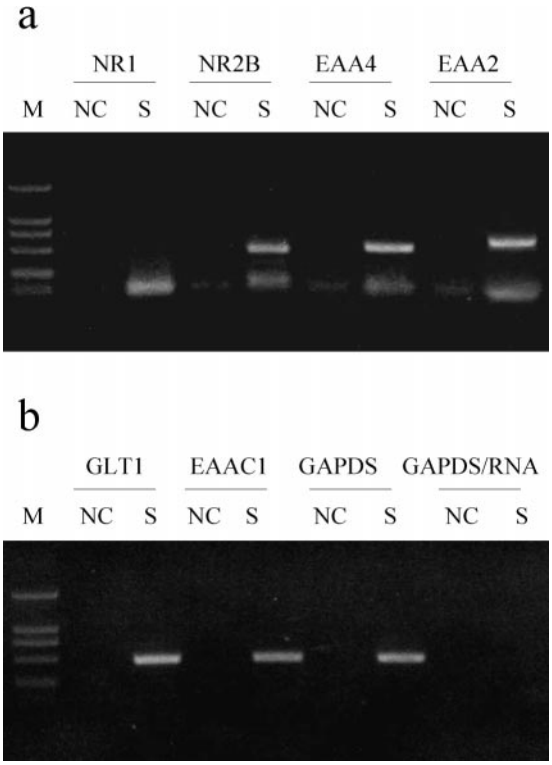

Figure 5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression of glutamate transporters and receptors in human sperm (S). M indicates DNA marker (2000, 1000, 750, 500, 250, 100 bp). **(a)** Indicates the amplification of cDNA of glutamate receptors (NR1, NR2B, EAA4 (GluR6), EAA2 [KA2]) with RT-conducted RNA samples using selective primers. **(b)** Indicates the amplification of glutamate transporters (GLT1, EAAC1) with RT-conducted RNA samples using selective primers. Glyceraldehyde-3-phosphate dehydrogenase testis specific (GAPDS) cDNA served as an internal standard and the direct amplification with RT-omitted RNA samples was conducted using GAPDS primers. NC indicates RNA-omitted PCR as negative control.

ysis (Figure 4a and b). Specific fragments from mouse sperm 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 by sequencing analysis. It was found that the products from mouse sperm are identical to those from mouse brain. GAPDH cDNA served as a control of RT samples (Figure 4b). In addition, no specific PCR product from the RNA samples that were omitted in reverse transcription could be observed over background, which verified the absence of genomic DNA contamination (Figure 4b). The data suggested that glutamate receptors and transporters were always expressed, which provided evidence that there existed those genes in mouse sperm.

RT-PCR in Human Sperm

Because the glutamate system was present in mouse sperm, we wondered whether it existed in human sperm. Selective primers were designed for the human genes identical to those mouse genes and RT-PCR analysis was performed. Results revealed that NR2B, EAA4 (GluR6), EAA2 (KA2), GLT1, and EAAC1 were detected in human sperm, whereas no NR1 was detected (Figure 5a and b). Testis-specific GAPDS convinced the origin of RNA and absent genomic DNA contamination (Figure 5b). The data suggested that glutamate receptors and transporters were always expressed, which provided evidence that there existed those genes in human sperm.

Discussion

Several laboratories reported the presence of GABA receptors and transporters in testis and sperm (Akinci and Schofield, 1999; Hu et al, 2000; Ma et al, 2000; He et al, 2001) and an important function of GABA in the process of sperm maturation was demonstrated. In the present study, we provided the first evidence that receptors (NR1, NR2B, GluR6, and KA2) and glutamate transporters (GLT1 and EAAC1) also existed in sperm. As we know, the CNS and male reproductive system possess blood barriers (blood-brain barrier and blood-testis barrier) and also need high input concentrations of blood glucose. This phenomenon is noteworthy and interesting, but up to now, its mechanism has been unclear. Our findings made the issue of similarity in the gene expression pattern between the CNS and the male reproductive system more intriguing.

Glutamate uptake inhibitors could block glutamate uptake in mouse sperm, and THA (nonselective inhibitor) was more potent as an inhibitor than DHK (GLT1-selective inhibitor) was (Figure 3a), suggesting that there were other glutamate transporters in sperm in addition to GLT1. Recent studies revealed that there was no specific immunostaining for EAAC1 in spermatozoa, but EAAC1 was detected in testis using RT-PCR and immunohistochemistry (Wagenfeld et al, 2002). It is possible that the expression of EAAC1 in sperm is only at the RNA level. The different distribution pattern of glutamate, GLT1, and NR2B found in mouse sperm indicated that they might play different roles in the function of sperm. NR2B was located on the tail of sperm (Figures 1b and 2b), the distribution of which was similar to type-5 metabotropic receptor of glutamate (mGluR5) localized in the midpiece and tail (Storto et al, 2001), which suggested that NR2B could be active in regulating sperm motility. As GABA could increase human sperm motility (Calogero et al, 1996) and induce the acrosome reaction in human spermatozoa (Shi et al, 1997), the glutamate system might also play a role in reproduction.

Taken together, we first demonstrated that the glutamate system was present in mouse and human sperm. All findings mentioned lend strong support to the conclusion that

glutamate might have unknown effects in sperm other than as the precursor of GABA or as a neurotransmitter in the brain. Because the location of glutamate, GLT1, and NR2B in human sperm (Figure 2a through c) is similar to that in mouse sperm, respectively (Figure 1a through c), we propose that glutamate receptors and transporters can be therapeutically targeted for contraception or dysgenesis treatment. We are also awaiting a deeper understanding of the mechanisms and functions of glutamate receptors and transporters in male reproductive tissues.

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