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# Possible expression of functional glutamate transporters in the rat testis

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## Abstract

Neither expression nor functionality is clear in peripheral tissues with the molecular machineries required for excitatory neurotransmitter signaling by L-glutamate (Glu) in the central nervous system, while a recent study has shown that several Glu receptors are functionally expressed in the rat testis. This fact prompted us to explore the possible functional expression in the rat testis of the Glu transporters usually responsible for the regulation of extracellular Glu concentrations in the brain. RT-PCR revealed the expression, in the rat testis, of mRNA for five different subtypes of Glu transporters, in addition to that for particular subtypes of ionotropic and metabotropic Glu receptors. Glutamate transporter-1 (GLT-1) was different in the brain from that in the testis in terms of molecular sizes on Northern and Western blot analyses. *In situ*

hybridization as well as immunohistochemical analysis showed localized expression of glutamate aspartate transporter at interstitial spaces and GLT-1 at elongated spermatids in the rat testis respectively. The expression of mRNA was localized for excitatory amino acid transporter-5 at the basal compartment of the seminiferous tubule in the rat testis. [<sup>3</sup>H]Glu was accumulated in testicular crude mitochondrial fractions in a temperature- and sodium-dependent saturable manner with pharmacological profiles similar to those shown in brain crude mitochondrial fractions. These results suggested that particular subtypes of central Glu transporters for the regulation of extracellular Glu concentrations in the rat testis could be constitutively and functionally expressed.

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## Introduction

L-Glutamate (Glu) is believed to play an excitatory amino acid neurotransmitter role through particular molecular machineries required for signaling processes in the central nervous system (CNS). These include Glu receptors (GluRs) for signal input, vesicular Glu transporters (VGLUTs) for signal output and Glu transporters (GluTs) for signal termination. In the CNS, extracellular Glu could contribute to the mechanism associated with synaptic plasticity, with neurotoxicity at an excessive level. GluTs are required for the termination of signal transduction mediated by Glu as well as for the prevention of neurotoxicity mediated by this endogenous excitotoxin. These transporters sometimes maintain a 10 000-fold gradient of intracellular Glu (3–10 mM) to extracellular Glu (0.3–1 μM), which is driven by the ionic gradients generated by ion-exchanging pumps such as Na<sup>+</sup>/K<sup>+</sup>-ATPase (Schousboe & Divac 1979, Danbolt 2001). To date, GluTs have been classified into five different subtypes, including glutamate aspartate transporter (GLAST) (excitatory amino acid transporter 1; EAAT1), glutamate transporter-1 (GLT-1) (EAAT2), excitatory amino acid carrier (EAAC1, EAAT3), EAAT4 and EAAT5 (Danbolt

2001). Both GLAST and GLT-1 are found primarily in glia cells, while EAAC1 is widely distributed in neurons. EAAT4 and EAAT5 are selectively expressed in the cerebellum and retina respectively. All these GluTs are coupled to the electrochemical gradient of sodium ions across cell membranes, with co-transport of one proton molecule and counter-transport of one potassium molecule (Danbolt 2001).

There is accumulating evidence in the literature for the expression of particular subtypes of GluRs and GluTs outside the CNS. In bone, for instance, both *in situ* hybridization and immunohistochemical analyses have revealed the expression of GLAST in both osteoblasts and osteoclasts (Mason *et al.* 1997, Huggett *et al.* 2000), in addition to the expression of various subunits and subtypes of ionotropic GluRs (iGluRs) (Chenu *et al.* 1998, Patton *et al.* 1998) and metabotropic GluRs (mGluRs) (Gu & Publicover 2000, Hinoi *et al.* 2001, 2002*b*). In adrenal glands expressing mRNA for particular subunits of iGluRs (Hinoi *et al.* 2002*a*), moreover, the expression of GLAST has been shown on Northern and Western blot analyses (Lee *et al.* 2001). GluTs could therefore play a role as the molecular machinery responsible for either the termination

of signal transduction or the prevention of toxicity mediated by extracellular Glu in bone and adrenal glands as well as in the CNS.

In the rat testis, mRNA and the corresponding proteins are constitutively and functionally expressed for both mGluR1 and mGluR5, but not for mGluR2 and mGluR3 (Storto *et al.* 2001). An agonist for group I mGluR subtype markedly facilitates hydrolysis of inositol phospholipid in slices of rat testis (Storto *et al.* 2001). Immunohistochemical and molecular biological analyses have revealed the expression of NMDA receptor (NR1) in germinal epithelium and interstitial spaces, GluR2/3 in interstitial spaces, mGluR2/3 proteins in interstitial cells and the wall of arterioles, mGluR1 in Sertoli cells and mGluR5 in germinal cells except spermatogonia (Gill *et al.* 2000, Storto *et al.* 2001). For signal transduction mediated by Glu in the testis, however, Glu should be incorporated in adjacent cells for the termination of Glu signals through GluTs with high affinity, in addition to the receptor system as described above. A Northern blot study has shown expression of mRNA for a novel GluT later found to be identical to GLAST (Storck *et al.* 1992, Danbolt 2001) in rat forebrain, cerebellum and testis (Tanaka 1993). Expression of mRNA has also been shown for the neuron-specific glutamate aspartate transporter isolated from human brain cDNA libraries in human brain, liver, muscle, ovary and testis on Northern blot analysis (Shashidharan *et al.* 1994). In order to elucidate a possible role of Glu as a signal mediator in the testis, therefore, we have attempted to examine the possible expression and localization of particular subtypes of GluTs, in addition to GluRs, in the rat testis using different molecular biological techniques.

## Materials and Methods

### Materials

[ $\alpha$ - $^{32}$ P]dCTP (111 TBq/mmol) and [ $^3$ H]L-Glu (1587.3 GBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). Guinea pig polyclonal antibodies against GLAST (carboxy terminus) and GLT-1 (carboxy terminus) were obtained from CHEMICON International (Temecula, CA, USA). A rabbit anti-rat EAAT4 antibody (carboxy terminus) was purchased from Alpha Diagnostic International (San Antonio, TX, USA). A biotinylated anti-guinea pig IgG antibody and VECTASTAIN Elite ABC Reagent were provided by Vector Laboratories (Burlingame, CA, USA). QuickPrep Micro mRNA Purification Kit, Ready-To-Go You-Prime First-Strand Beads, anti-guinea pig IgG antibody conjugated with horseradish peroxidase, ECL detection reagent and Rediprime II random prime labeling system were supplied by Amersham Biosciences (Piscataway, NJ, USA). Taq polymerase was obtained from Takara (Shiga, Japan). ISOGEN, D-glutamate, L-aspartate and kainic

acid (KA) were purchased from Wako (Osaka, Japan). Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns and Bio-Rad Protein Assay Kit were provided by Bio-Rad Laboratories (Hercules, CA, USA). Lig'nScribe No-Cloning Promoter Addition Kit was supplied by Ambion (Austin, TX, USA). Digoxigenin (DIG) RNA Labeling Kit (SP6/T7) and anti-DIG-AP Fab fragments were obtained from Roche Diagnostics (Mannheim, Germany). L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), L-(−)-threo-3-hydroxyaspartic acid (THA), (2S,1'S,2'R)-2-(carboxycyclopropyl)glycine (CCGIII), N-methyl-D-aspartic acid (NMDA), DL- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), (RS)-3,5-dihydroxyphenylglycine (DHPG), (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCGIV) and L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) were purchased from Tocris (Bristol, UK). Other chemicals used were all of the highest purity commercially available. Whatman GF/B glass fibre filter was obtained from Whatman (Clifton, NJ, USA).

### Animals

The protocol employed in this study meets the guideline for animal experimentation of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. Male Wistar rats at 7 weeks of age were purchased from a local supplier and housed in our breeding rooms at 25 °C with a humidity of 55% and a light:darkness cycle of 12 h, with free access to water and food.

### RT-PCR

Each tissue was subjected to extraction of mRNA using the QuickPrep Micro mRNA Purification Kit, followed by the synthesis of DNA with 12.5  $\mu$ M random hexamer primers and Ready-To-Go You-Prime First-Strand Beads as described previously (Hinoi *et al.* 2001). A reaction with reverse transcriptase was run at 37 °C for 1 h, and an aliquot of synthesized cDNA was used directly for PCR conducted in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP, 20 pmol of each primer and 0.625 units Taq polymerase. Cycling conditions used for 40 PCR cycles were as follows: GLAST, GLT-1, EAAC1, EAAT4 and EAAT5, denaturation for 45 s at 95 °C, annealing for 40 s at 64 °C and extension for 40 s at 72 °C. Electrophoresis was run for an aliquot of PCR amplification products on a 2% agarose gel, followed by detection of DNA with ethidium bromide. Appropriate PCR DNA products were extracted from agarose gel using DNA extraction spin columns, followed by sequencing by ABI Prism 310

Genetic Analyzer (Perkin Elmer) using a cycle sequencing kit.

#### Western blotting

Each tissue was homogenized in 10 mM HEPES–NaOH buffer (pH 7.9) containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, 10 mM sodium fluoride, 10 mM sodium  $\beta$ -glycerophosphate and 1  $\mu$ g/ml of each protease inhibitor (*p*-aminophenyl methanesulfonyl fluoride, benzamide, leupeptine, antipain), followed by centrifugation at 800 *g* for 10 min. Supernatants were centrifuged at 20 000 *g* for 20 min, and the resultant supernatants were centrifuged at 100 000 *g* for 60 min. The pellets thus obtained (P3 fractions) were suspended in the aforementioned buffer and the suspensions were mixed at a volume ratio of 4:1 with 10 mM Tris–HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 0.01% bromophenol blue and 5% mercaptoethanol, followed by boiling at 100 °C for 10 min. Each aliquot of 0.2 or 20  $\mu$ g protein was subjected to electrophoresis on a 10% polyacrylamide gel at a constant current of 15 mA/plate for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After being blocked by 5% skimmed milk dissolved in 20 mM Tris–HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20 (TBST), the membrane was incubated with guinea pig polyclonal antibodies against GLAST (1:8000 dilution) and GLT-1 (1:4000 dilution) or rabbit polyclonal antibody against EAAT4 (1:1600 dilution) diluted with TBST containing 1% skimmed milk, and then incubated with the anti-guinea pig IgG antibody or the anti-rabbit antibody conjugated with horseradish peroxidase. Finally, the membrane was incubated with ECL detection reagent, followed by exposure to X-ray films for different periods to obtain the most appropriate blots.

#### Northern blotting

Each tissue was subjected to extraction of total RNA using the ISOGEN. An aliquot of total RNA (neocortex, 5 or 10  $\mu$ g/lane; retina, 5  $\mu$ g/lane; testis, 50  $\mu$ g/lane) was subjected to electrophoresis on a 1% denatured agarose gel containing 2.2 M formaldehyde at a constant voltage of 50 V at room temperature and subsequently transferred to a nylon membrane by capillary blotting. The blotted membrane was previously hybridized with 5  $\times$  SSC (750 mM NaCl, 75 mM sodium citrate) containing 50% formamide, 5  $\times$  Denhardt's, 0.5% SDS and 25  $\mu$ g/ml salmon sperm DNA at 42 °C and then hybridized with <sup>32</sup>P-radiolabeled fragments of the cDNA at 42 °C for 16 h. The cDNA probe was prepared by RT-PCR with primers specific for GLT-1 or EAAT5, followed by isolation of the amplified product on the gel and subsequent purification by Quantum Prep Freeze 'N Squeeze

DNA Gel Extraction Spin Columns for radiolabeling with [ $\alpha$ -<sup>32</sup>P]dCTP using the Rediprime II random prime labeling system. The hybridized membrane was successively washed with 2  $\times$  SSC containing 0.1% SDS at room temperature for 10 min twice, 1  $\times$  SSC containing 0.1% SDS at 42 °C for 20 min, and then 0.5  $\times$  SSC containing 0.1% SDS at 42 °C for 20 min, followed by exposure to X-ray films for different periods to obtain the most appropriate blots.

#### Preparation of DIG-labeled cRNA probe

The cDNA fragments were amplified using the PCR procedures described above, followed by ligation reaction with the T7 promoter adapter using the Lig'nScribe No-Cloning Promoter Addition Kit. An aliquot of synthesized T7 promoter-adapted cDNA was used directly for PCR carried out in buffer containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP, 6.25 pmol PCR adapter primer (5'-GCTTCCGGCTCGTATGTTGTGTGG-3') and gene specific primer (to produce the antisense (sense) cRNA probe, 5' (3') gene specific primer) and 1.25 units Taq polymerase. The cycling conditions used were as follows: 30 cycles of 30 s at 94 °C for denaturation, 30 s at 57 °C for annealing and 1 min at 72 °C for extension. Subsequently, to produce DIG-labeled cRNA, amplified T7 promoter-adapted cDNA was transcribed by T7 RNA polymerase according to the manufacturer of the DIG RNA Labeling Kit (SP6/T7). The DIG-labeled cRNA was stored at –20 °C until use.

#### In situ hybridization

Testes were removed from male Wistar rats, followed by rapid freezing on crushed dry ice and subsequent dissection of sections at a thickness of 10  $\mu$ m in a cryostat at –20 °C. These sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 20 min, followed by washing three times with 0.1 M PB, treating with 0.2 M HCl for 10 min, washing three times with 0.1 M PB, treating with 10  $\mu$ g/ml proteinase K for 5 min and washing three times with 0.1 M PB. Sections were then subjected to acetylation in 0.1 M triethanolamine/0.25% acetic anhydride for 10 min, followed by washing with 0.1 M PB and stepwise dehydration in 70, 80, 90, 95 and 100% ethanol for 5 min each. After being dried, sections were covered with the hybridization buffer (10% dextran sulfate, 5  $\times$  SSC, 20 mM Tris–HCl (pH 8.0), 300 mM NaCl, 50% formamide, 1  $\times$  Denhardt's and 500  $\mu$ g/ml yeast tRNA) containing 25  $\mu$ g/ml salmon sperm DNA at 65 °C for 1 h, and then incubated with hybridization buffer containing the DIG-labeled cRNA probe at 65 °C for 16 h. Post-hybridization washes were done stepwise with 4  $\times$  SSC at 65 °C for 20 min, 50% formamide in 2  $\times$  SSC at 65 °C for

30 min, TNE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 500 mM NaCl) at 37 °C for 10 min three times, 4 µg/ml RNase A in TNE buffer at 37 °C for 30 min, TNE buffer at 37 °C for 10 min, 2 × SSC at 65 °C for 30 min, 0.2 × SSC at 65 °C for 30 min, buffer 1 (100 mM Tris-HCl (pH 7.5) and 150 mM NaCl) at room temperature for 10 min and then 1.5% blocking buffer (1.5% blocking reagent in buffer 1) at room temperature for 1 h. Subsequently, sections were washed with buffer 1 at room temperature, and then incubated with 0.75 U/ml anti-DIG-AP Fab fragments in 0.5% blocking buffer containing 0.2% Tween at 4 °C for 16 h. After being washed four times with buffer 1 containing 0.2% Tween at room temperature for 15 min, sections were treated with buffer 2 (100 mM Tris, 100 mM NaCl and 50 mM MgCl<sub>2</sub> (pH 9.5)) and then developed in buffer 2 containing 375 µg/ml nitro blue tetrazolium chloride and 188 µg/ml 5-bromo-4-chloro-3-indolyl phosphate for different periods to obtain the most appropriate pictures. After washing with buffer 2 at room temperature for 5 min, the development was stopped by incubation in 1 × TE (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0)).

#### Immunohistochemistry

Testes were removed from male Wistar rats, followed by rapid freezing on crushed dry ice and subsequent dissection of sections at a thickness of 10 µm in a cryostat at -20 °C. Sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, followed by washing with PBS, treating with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min and washing with 70% ethanol for 5 min. After being washed with PBS, sections were subjected to blocking with PBS containing normal goat serum and 0.1% Triton X-100 at room temperature for 1 h. Sections were then reacted with antibodies against GLAST (1:500 dilution) and GLT-1 (1:400 dilution) diluted with the same blocking buffer at room temperature for 1 h, followed by reaction with biotinylated anti-guinea pig IgG antibody at room temperature for 30 min and subsequent incubation with VECTASTAIN Elite ABC Reagent at room temperature for 1 h. Finally, immunostaining was carried out using 0.05% diaminobenzidine and 0.01% hydrogen peroxide using hematoxylin as the counterstain.

#### [<sup>3</sup>H]Glu uptake assay

For evaluation of the functionality of the GluTs expressed, an attempt was made to determine whether rat testis indeed has the ability to accumulate radiolabeled substrate in a temperature-dependent saturable manner. Testes obtained from male Wistar rats at 7 weeks of age were rapidly homogenized in 0.32 M sucrose and homogenates were centrifuged at 800 g for 20 min. The resultant supernatants were centrifuged at 20 000 g for 20 min. The

pellets thus obtained (P2) were suspended in 0.32 M sucrose and centrifuged at 20 000 g for 20 min again. These washing procedures were repeated three times, and the final pellets were suspended in HEPES buffer (125 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 10 mM HEPES and 10 mM D-glucose (pH 7.4)) for use in the uptake experiments (Takarada *et al.* 2003). Membrane suspensions were preincubated for 5 min at 2 °C or 30 °C, followed by incubation with 10 nM [<sup>3</sup>H]L-Glu for 1–60 min. For pharmacological analysis, test compounds were introduced into the incubation medium at the beginning of the preincubation. The incubation was terminated by the addition of ice-cold buffer at 2 °C and subsequent filtration through a Whatman GF/B glass fiber filter under constant vacuum. The filter was rinsed four times with ice-cold buffer at 2 °C, and the radioactivity retained on the filter was measured using a liquid scintillation spectrometer. Assays were always carried out in triplicate with variations of less than 10%. Protein concentration was determined with Bio-Rad protein.

#### Data analysis

Results are all expressed as the means ± S.E. and statistical significance was determined by two-tailed and unpaired Students' *t*-test or ANOVA with Bonferroni/Dunnett post hoc tests.

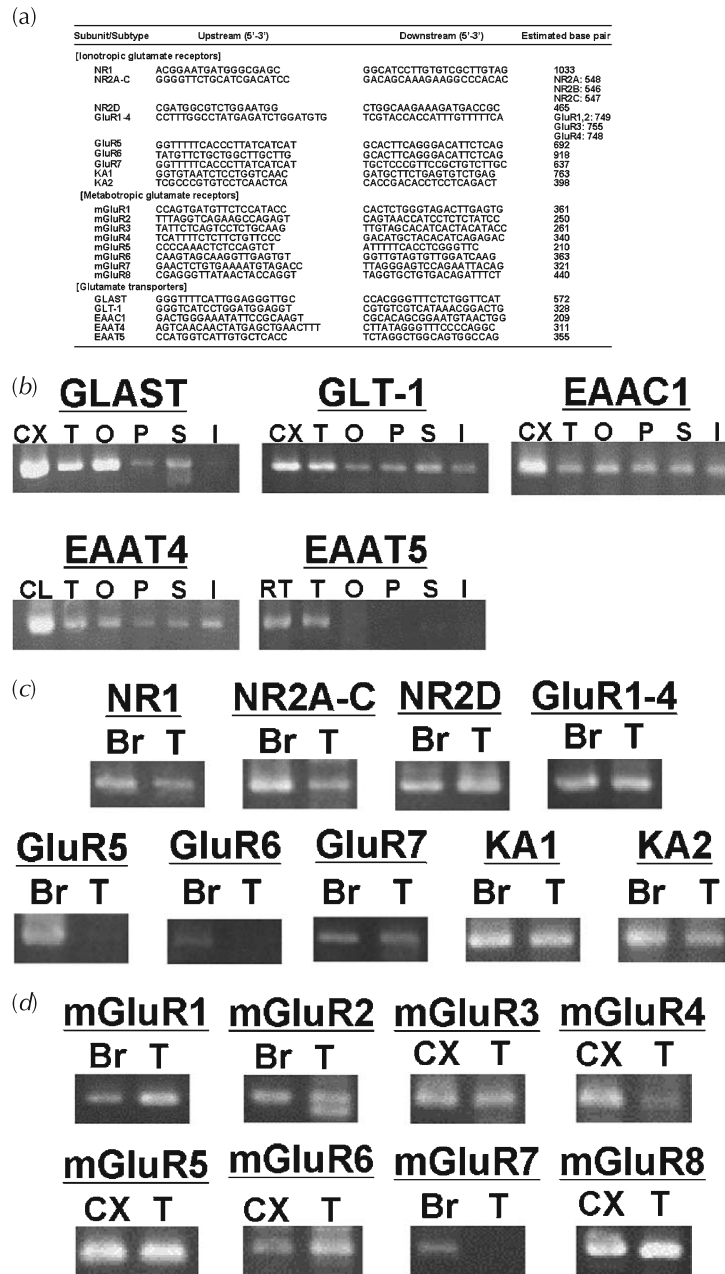
## Results

#### Expression of mRNA

Figure 1a shows the nucleotide sequences of the primers used for the demonstration of expression of mRNA for iGluRs, mGluRs and GluTs in this study. Tissue samples were homogenized, followed by extraction of mRNA and subsequent RT-PCR using one of these primers. Figure 1b shows profiles of the expression of mRNA for five different GluTs cloned to date in different tissues of the rat on RT-PCR. GLAST mRNA was expressed in rat neocortex, testis, ovary, pancreas and stomach but not in intestine, while GLT-1 mRNA was invariably expressed in all tissues tested. Similarly, mRNA for both EAAC1 and EAAT4 was expressed in neocortex or cerebellum, testis, ovary, pancreas, stomach and intestine. However, mRNA was expressed for EAAT5 in both retina and testis only, but not in other regions including ovary, pancreas, stomach and intestine.

In rat whole brain, mRNA was expressed for all iGluR subtypes tested (Fig. 1c). These included NR1, NR2A–C and NR2D subunits for NMDA receptors, GluR1–4 and GluR5 subunits for AMPA receptors, and GluR6, GluR7, KA1 and KA2 subunits for KA receptors respectively. In the rat testis, by contrast, mRNA was not expressed for

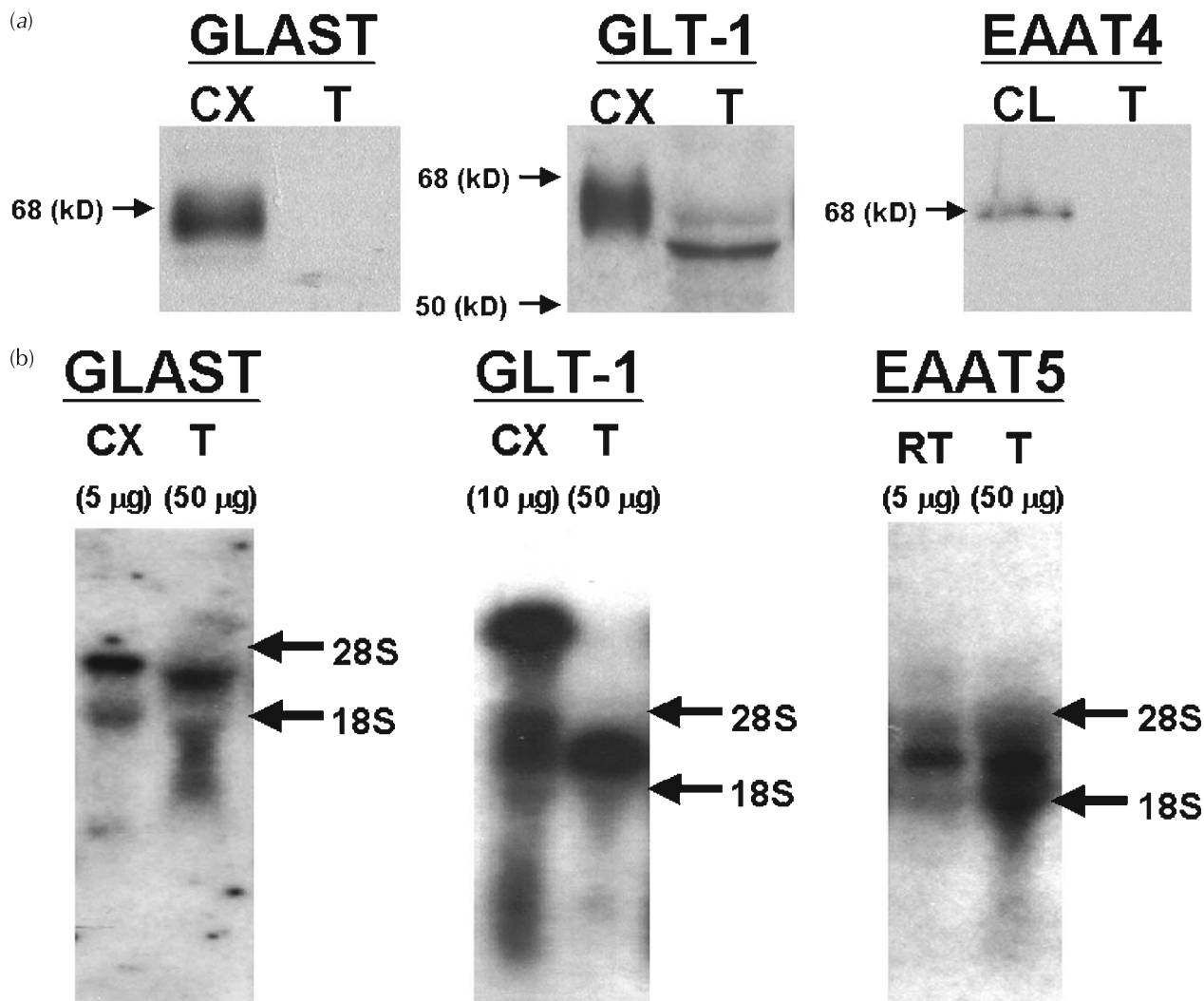




**Figure 1** RT-PCR analysis on GluTs and GluRs. (a) Nucleotide sequences of primers used. mRNA was extracted from different tissues of the rat, followed by RT-PCR using specific primers for (b) GluTs, (c) iGluRs and (d) mGluRs. The experiments were repeated at least three times using different animals with similar results. CX, neocortex; CL, cerebellum; RT, retina; T, testis; O, ovary; P, pancreas; S, stomach; I, intestine; Br, whole brain.

either GluR5 or GluR6 subunits of KA receptors with expression of mRNA for other iGluR subunits examined. In rat whole brain or neocortex, mRNA was invariably expressed for all mGluRs tested, including mGluR1 to

mGluR8 (Fig. 1d). Of the mGluRs examined, however, mRNA was expressed for mGluR1 to mGluR6 and mGluR8 but not for mGluR7 in rat testis. In particular, two splicing variants were detected with mRNA for



**Figure 2** Western and Northern blot analyses on GluTs. (a) Microsomal (P3) fractions were prepared from each tissue, followed by SDS-PAGE and subsequent detection of immunoreactive GLAST, GLT-1 and EAAT4. (b) Total RNA was extracted from each tissue, followed by electrophoresis on agarose gel and subsequent incubation with [ $^{32}$ P]labeled cDNA probe for GLAST, GLT-1 or EAAT5. The experiments were repeated at least three times using different animals with similar results. CX, neocortex (0.2 µg in (a)); CL, cerebellum (0.2 µg in (a)); T, testis (20 µg in (a)); RT, retina.

mGluR2 in rat testis in contrast to one single mGluR2 mRNA in rat whole brain.

#### Expression of GluTs

Rat neocortex, cerebellum and testis were homogenized in buffer containing different protease inhibitors, followed by successive centrifugation to obtain microsomal (P3) fractions, and subsequent SDS-PAGE for immunoblotting analysis using commercially available antibodies against GLAST, GLT-1 and EAAT4. Immunoreactivity was found with GLT-1 (Fig. 2a, middle panel) in the rat testis at a molecular weight position smaller than that for

the neocortex, but not with GLAST (Fig. 2a, left panel) and EAAT4 (Fig. 2a, right panel), under the conditions employed (Fig. 2a). Immunoblotting analysis using the secondary antibody alone led to detection of weak immunoreactivity at a position similar to cortical GLT-1 in the rat testis, but not in the neocortex (data not shown).

To further analyze the difference in molecular size of GLT-1 between neocortex and testis on Western blot analysis, Northern blot analysis was carried out using a radiolabeled cDNA probe for GLT-1 mRNA. The GLT-1 cDNA probe was prepared by RT-PCR with primers specific for GLT-1, followed by isolation of amplified product on the gel and subsequent purification

for radiolabeling with [ $\alpha$ - $^{32}$ P]dCTP using a labeling system. Total RNA was extracted for Northern blot analysis from rat neocortex and testis. As shown in Fig. 2b, a clearly smaller size was seen with mRNA detected by the radiolabeled cDNA probe for GLT-1 in total RNA extracts from testis than those from neocortex detected as an upper radioactive band at the position demonstrated previously (Fig. 2b, middle panel), with a slightly smaller size for GLAST mRNA in testis than in neocortex (Fig. 2b, left panel). By contrast, radioactive bands were found at almost similar molecular size positions with mRNA for EAAT5 between total RNA extracts prepared from retina and testis (Fig. 2b, right panel).

#### Localization of GluTs

In Fig. 3a, a schematic representation is shown for the histological characteristics of the testis. Rat testis was removed, followed by dissection of frozen sections and subsequent fixation with paraformaldehyde for detection of histological localization using DIG-labeled cRNA probe. *In situ* hybridization evaluation clearly demonstrated the localized expression of mRNA for GLAST (Fig. 3b, left panels) at the interstitial space and for GLT-1 (Fig. 3b, second left panels) at the elongated spermatid in the rat testis respectively (Fig. 3b). Further *in situ* hybridization analysis was carried out with cRNA probes directed against other GluT subtypes including EAAC1, EAAT4 and EAAT5 using frozen sections of rat testis. Compared with negative control sections reacted with individual sense cRNA probes, expression of EAAT5 mRNA was found in the basal compartment of seminiferous tubules in the rat testis (Fig. 3b, right panels), but mRNA expression was not seen for EAAC1 and EAAT4 in the rat testis. However, specification of the exact location of expression of mRNA for EAAT5 in the basal compartment comprised of spermatocytes, Sertoli cells and spermatogonia in the rat testis was not accomplished.

In order to confirm the localized expression of particular subtypes of GluTs in rat testis, immunohistochemical analysis was next made using commercially available antibodies against GLAST and GLT-1 on frozen sections of rat testis. A similar localization profile was seen with immunoreactive GLAST in the interstitial space on immunohistochemistry using an antibody against GLAST in the rat testis (Fig. 3c, left panels), while immunoreactive GLT-1 was also found at elongated spermatids in the rat testis as seen on *in situ* hybridization (Fig. 3c, right panels).

#### [ $^3$ H]Glu uptake

In order to elucidate a part of the functionality of the GluTs expressed, an attempt was next made to determine whether rat testis is really able to incorporate a substrate in a temperature-dependent saturable manner as seen in the

brain. Rat testes were homogenized, followed by preparation of crude mitochondrial fractions and subsequent determination of the accumulation of [ $^3$ H]Glu using a rapid filtration method. The accumulation of [ $^3$ H]Glu rapidly increased with incubation time at 30 °C in a temperature-dependent manner and reached a plateau within 30 min (Fig. 4a). Elevation of incubation temperature from 2 °C to 30 °C more than doubled the accumulation of [ $^3$ H]Glu when determined at 60 min, while the accumulation also increased with incubation time even at 2 °C, with a plateau within 30 min.

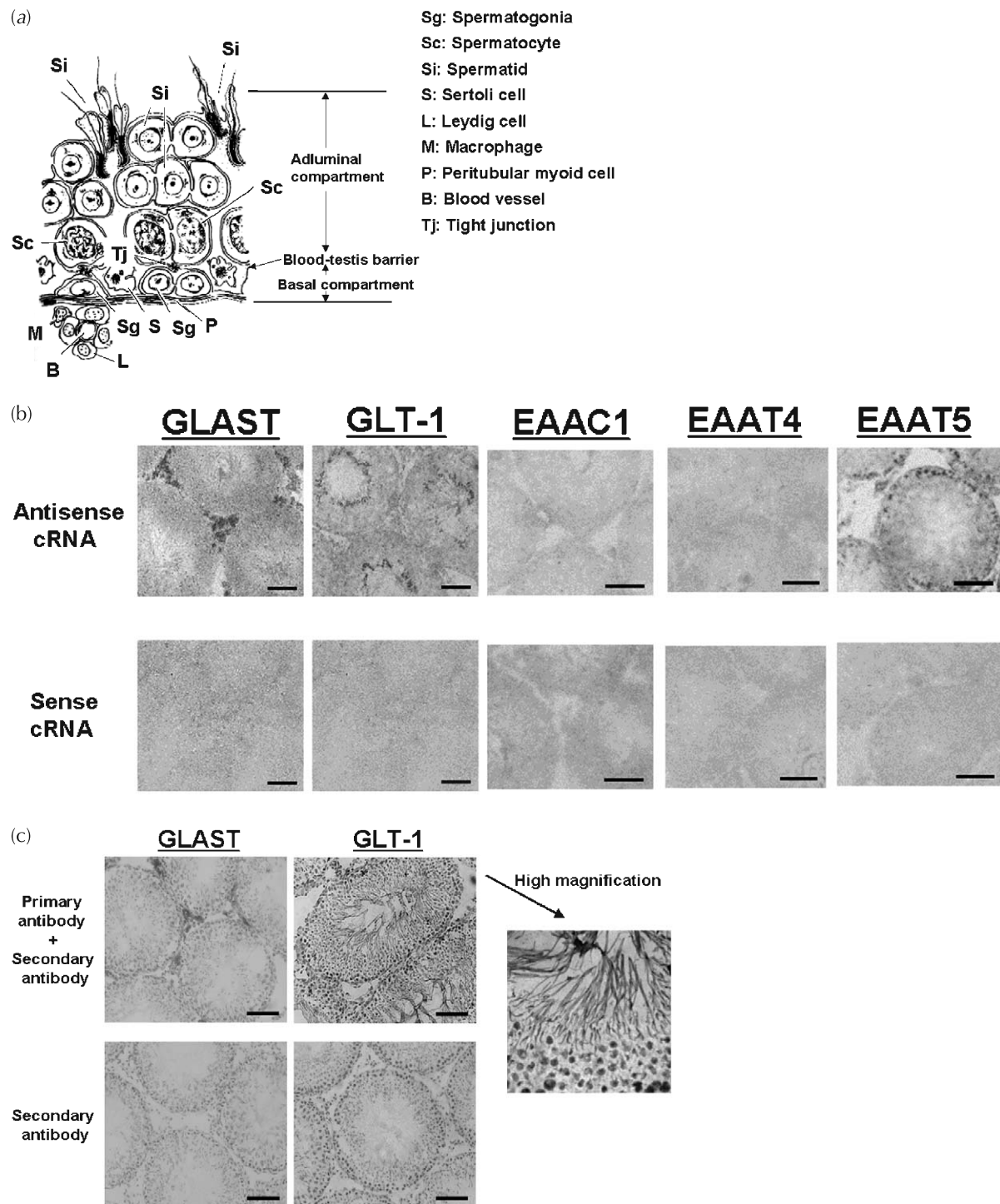
Rat testicular fractions were incubated with [ $^3$ H]Glu in buffer containing different compounds at 10–100  $\mu$ M at 30 °C for 50 min. [ $^3$ H]Glu accumulation was inhibited by L-Glu, L-Asp, D-Asp, PDC, THA and CCGIII in a concentration-dependent manner at a concentration range of 10–100  $\mu$ M, with no significant inhibition by D-Glu, NMDA, AMPA, KA, DHPG, DCGIV, L-AP4 and L-alanine at 100  $\mu$ M (Fig. 4b). Rat testicular fractions were incubated with different concentrations of [ $^3$ H]Glu at concentrations of up to 30  $\mu$ M at 30 °C for 10 min for determination of saturation isotherms. The accumulation increased with increasing concentrations of [ $^3$ H]Glu followed by saturation at 30  $\mu$ M (Fig. 4c). Woolf-Hanes plot analysis on [ $^3$ H]Glu accumulation in rat testicular fractions revealed a single component with a  $K_m$  value of  $24.2 \pm 4.4$   $\mu$ M and a  $V_{max}$  value of  $85.7 \pm 12.8$  pmol/mg protein per min respectively (Fig. 4d).

In order to confirm the net incorporation of radiolabeled substrate, membrane fractions were treated with 0.01% Triton X-100 and then incubated with [ $^3$ H]Glu at 30 °C for 50 min. As shown in Fig. 4e, [ $^3$ H]Glu accumulation was significantly reduced to the level found at 2 °C in rat testicular fractions previously treated with Triton X-100. For evaluation of sodium dependency, rat testicular fractions were incubated with [ $^3$ H]Glu at 30 °C for 50 min in HEPES buffer with replacement of NaCl by equimolar ChCl. As shown in Fig. 4f, replacement of sodium chloride with choline chloride significantly decreased [ $^3$ H]Glu accumulation in rat testicular fractions to the level found at 2 °C.

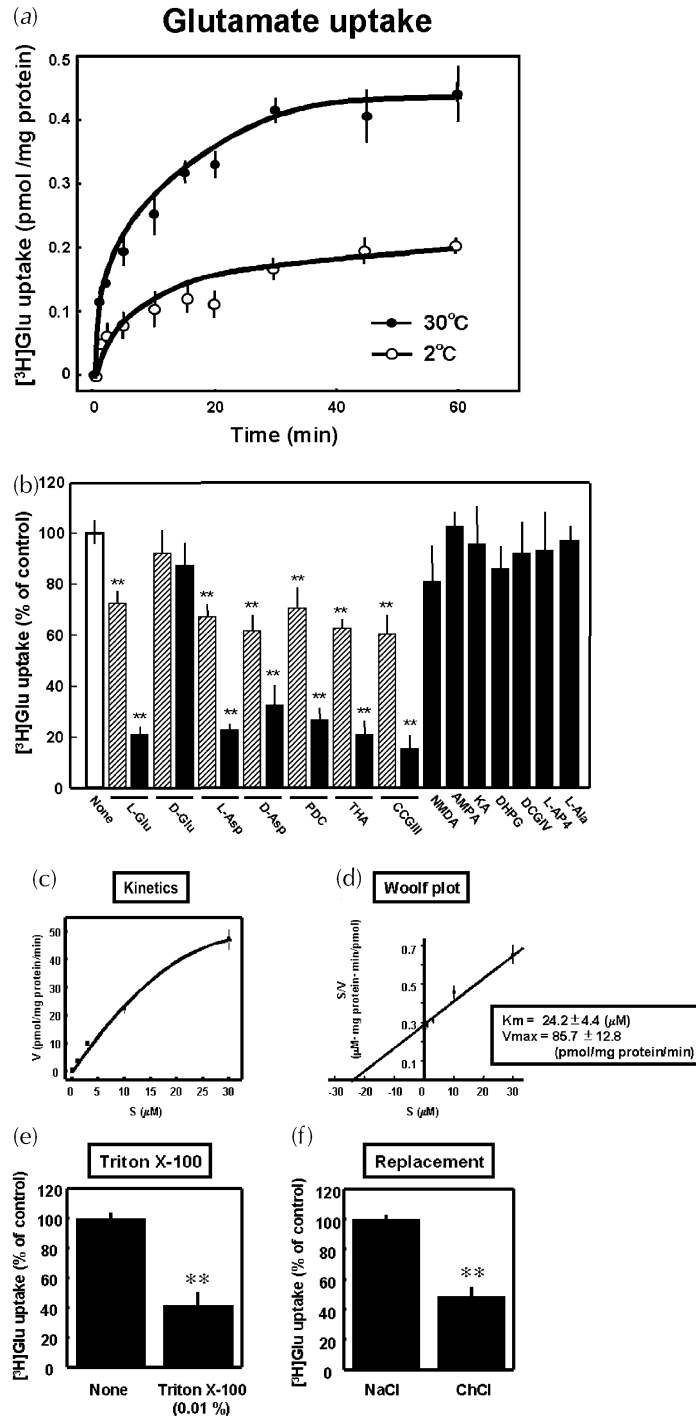
#### Discussion

The essential importance of the present findings was that both mRNA and the corresponding proteins are expressed for GLAST and GLT-1 with high localization at particular individual locations in the rat testis. Testicular preparations also exhibited the ability to accumulate [ $^3$ H]Glu in a temperature- and sodium-dependent saturable manner with pharmacological profiles peculiar to GluTs in the CNS. A previous Northern blot study has demonstrated the expression of mRNA for GLAST in the rat testis as well as the brain, without specification of the exact location (Tanaka 1993). In the present study, moreover,





**Figure 3** Analyses on the localization of GluTs in the rat testis. (a) Schematic representation of the histological characteristics of the testis. Rat testicular frozen sections were fixed with paraformaldehyde and then incubated with (b) DIG-labeled cRNA probe for *in situ* hybridization on GLAST, GLT-1, EAAC1, EAAT4 and EAAT5 or (c) antibodies against GLAST and GLT-1 for immunohistochemistry. Sections were stained with hematoxylin afterwards as needed. Typical micrographs are shown with similar results in three independent experiments. Scale bars=100  $\mu$ m.



**Figure 4** Accumulation of [<sup>3</sup>H]Glu in rat testis. (a) Rat testis was homogenized and subjected to incubation with [<sup>3</sup>H]Glu at 2 °C or 30 °C for different periods up to 60 min. (b) Rat testicular fractions were incubated with [<sup>3</sup>H]Glu at 30 °C for 50 min in buffer containing each compound at 10 μM (shaded bars) to 100 μM (solid bars). (c) [<sup>3</sup>H]Glu accumulation was measured at substrate concentrations of 0.01, 0.1, 1, 3, 10 and 30 μM at 30 °C for 10 min. (d) Woolf plot of the data obtained from the experiment shown in (c). (e) Rat testicular fractions were treated with 0.01% Triton X-100, followed by determination of [<sup>3</sup>H]Glu accumulation at 30 °C for 50 min. (f) Rat testicular fractions were incubated with [<sup>3</sup>H]Glu at 30 °C for 50 min in the presence of 125 mM NaCl or ChCl. All values shown are the means ± s.e. obtained in three separate experiments done in triplicate. \*\**P* < 0.01 significantly different from the individual control values.

the expression was shown with mRNA for GLT-1, EAAC1, EAAT4 and EAAT5 in rat testis on RT-PCR. To our knowledge, this paper deals with the first direct demonstration of the expression of mRNA for all GluT subtypes cloned to date in the rat testis. The paradoxical detection of GLAST between Western and immunohistochemical analyses could be accounted for by taking into consideration the differential sensitivities of the experimental procedures employed for detection, in addition to the localized expression of the protein concerned. Western blot assay seems less sensitive than immunohistochemistry at detecting a particular antigenic protein expressed in a small amount at a localized and restricted area in the rat testis as shown here. A similar paradox is seen for the expression of EAAT4 on RT-PCR, Western blot and *in situ* hybridization analyses in the present study. Comprehensive evaluation would be required for the identification and demonstration of a particular subtype of GluRs, GluTs and/or VGLUTs expressed in a particular organ. One of the interesting findings obtained in this study was the expression of mRNA for EAAT5, which has been believed to be specifically localized in the retina (Danbolt 2001) and in the rat testis on RT-PCR, Northern blot and *in situ* hybridization analyses. Commercial availability of an antibody against EAAT5 is therefore a determinant of the promotion and stimulation of basic and clinical research on this GluT subtype in future studies.

Evidence is emerging for a role of Glu as an extracellular signal mediator in the autocrine and/or paracrine system, in addition to an excitatory amino acid neurotransmitter role in the CNS (Skerry & Genever 2001). By analogy with neurotransmission in the central glutamatergic synapses, the expression of GluTs could be essential for the termination of Glu signals through reduction of the extracellular concentration in mechanisms underlying possible signal transduction mediated by Glu in the testis. In the CNS, GluTs are also required for attenuation of neurotoxic properties of Glu as an endogenous excitotoxin. Whether Glu is toxic to testicular cells as seen in central neurons, however, remains to be elucidated. From this point of view, it should be noted that exposure to D-aspartate selectively stimulates testosterone synthesis after incorporation into cells, with that to L- and D-Glu, L-aspartate and L- and D-asparagine being ineffective, in cultured rat Leydig cells (Nagata *et al.* 1999). Nagata *et al.* (1999) have also claimed expression of GLAST mRNA in rat Leydig cells without providing any direct evidence. In the present study, by contrast, highly localized expression of both mRNA and the corresponding protein for GLAST in the testicular interstitial space where Leydig cells are enriched in addition to both blood vessels and macrophages has been shown for the first time. Our data from *in situ* hybridization and immunohistochemical analyses are thus in good agreement with the report described above. GLAST is highly enriched in astroglial cells in the

CNS (Danbolt 2001), whereas testicular Leydig cells are shown to express glial fibrillary acidic protein which is a marker protein of astroglia (Holash *et al.* 1993). In the CNS, astroglia are shown to contribute to the blood–brain barrier through extension of their foot processes onto microvessels that constitute the physical entity, in addition to secretion of a trophic factor to endothelial cells in microvessels. Leydig cells would be, at least in part, involved in the blood–testis barrier constituted by tight junctions between Sertoli cells in seminiferous tubules as well as by myoid cells through secretion of a trophic factor, as seen with astroglial cells for the blood–brain barrier (Holash *et al.* 1993).

In contrast to VGLUTs required for condensation of Glu in synaptic vesicles, all GluTs cloned to date have been shown to be unable to differentiate between optic isomers of aspartate (Danbolt 2001). D-Aspartate endogenously occurs in a rank order of decreasing levels, while an intraperitoneal injection of D-aspartate induces a marked increase in its endogenous levels in these neuroendocrine tissues including the testis (D'Aniello *et al.* 2000). These previous and present findings all give support to the idea that testosterone synthesis could be facilitated by D-aspartate incorporated into intracellular locations through GLAST expressed at Leydig cells in the testis. The possibility that GluTs could play a role crucial for signal transduction in mechanisms associated with intercellular communications within the testis is feasible so far. The present pharmacological evaluation failed to identify the participation of particular GluT subtypes in [<sup>3</sup>H]Glu accumulation in testicular mitochondrial fractions that were employed in analogy with brain crude mitochondrial fractions often used for uptake assays. The temperature-independent [<sup>3</sup>H]Glu accumulation seems to correspond, at least in part, to the portion insensitive to Triton X-100 and replacement with ChCl, whereas its physiological significance is unclear. Although [<sup>3</sup>H]Glu accumulation was not significantly inhibited by all agonists tested for iGluRs and mGluRs at 30 °C, the possible binding to particular subtypes of GluRs expressed in rat testis at 2 °C has not been ruled out so far. Pharmacological profiling could be made easier than that in the present analysis when [<sup>3</sup>H]Glu accumulation is determined under experimental conditions exclusively favorable for the temperature-dependent portion in the rat testis. In the CNS, five subtypes of GluTs show distribution profiles different from each other (Tanaka *et al.* 1997, Danbolt 2001). Lethal spontaneous seizures are seen in mice with the gene for GLT-1 knocked out (Tanaka *et al.* 1997), while GLAST-mutant mice show only slight motor discoordination without mortality (Watase *et al.* 1998). The present findings that GLAST, GLT-1 and EAAT5 exhibited different localization profiles in the rat testis as seen in the CNS argue in favor of the idea that each GluT subtype could play a role peculiar to individual subtypes

in the mechanisms associated with the maintenance of integration and function of different cells located in the rat testis.

In addition to the aforementioned five different subtypes of GluTs, this study has also demonstrated the expression of mRNA for particular iGluRs and mGluRs in the rat testis as revealed by RT-PCR. A previous study claims functional expression of both mGluR1 and mGluR5 for group I mGluR subtype in the rat testis (Storto *et al.* 2001), while the first evidence has been presented in the present investigation for the expression of additional mRNA of both mGluR2 and mGluR3 for the group II subtype as well as of both mGluR6 and mGluR8 for the group III subtype in the rat testis respectively, without expression of that of both mGluR4 and mGluR7 for the group III subtype. The expression of NR1, GluR2/3 and mGluR2/3 proteins has already been shown in the rat testis (Gill *et al.* 2000, Gill & Pulido 2001), whereas the present article seems to be the first direct evidence for the expression of mRNA of particular iGluRs, including NR2A-C, NR2D, GluR7, KA1 and KA2 subunits in the rat testis. Although the functionality of these particular GluR subunits demonstrated here should undoubtedly await further evaluation in future studies, the data cited above all argue in favor of the hypothesis that Glu could serve as a signal mediator through particular iGluR, mGluR and GluT subtypes expressed in the rat testis. For glutamatergic signal transduction, nevertheless, expression of VGLUTs as well as exocytotic release of Glu should be studied in the testis *in vivo* and *in vitro* as shown in cultured osteoblasts (Hinoi *et al.* 2001). Functional expression of molecular machineries required for glutamatergic signaling in the brain should be demonstrated in the rat testis before drawing any final conclusion.

It thus appears that Glu may be one of the endogenous substances affecting function and integrity through receptors and transporters in the rat testis. A search for receptor ligands as well as transporter modulators would be of great benefit for the discovery and development of drugs useful for the therapy and treatment of a variety of endocrinological disorders associated with testicular malfunction in human beings.

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