citrate buffer and 0.1% SDS at 60°C for 1 h. For northern blot analysis, 10 μg of total RNA from the rat cerebral neocortex was electrophoresed in 1% agarose gel containing 6.3% formaldehyde, then blotted onto a nylon membrane. After pre-hybidization at 65°C for 1 h, the blotted filter was hybridized with the ^{32}P-labelled antisense or sense RNA probe (probe 1) at 65°C for 12 h. The filter was washed in 0.1 × saline sodium citrate buffer and 0.1% SDS at 68°C for 1 h. In some experiments, pre-made RNA blots (Rat Multiple Tissue Northern (MTN™) Blot (BD Biosciences Clontech, Mountain View, CA, USA), and rat brain tissue blot, Seegene, Seoul, Korea) were used for northern hybridization with digoxigenin (DIG)-labelled RNA probe (probe 2), which was described in the *in situ* hybridization section in detail.

In situ hybridization

In situ hybridization was performed as previously described (Hoshino *et al.* 1999). Anaesthetized male Wistar rats at post-natal day 56 were fixed by perfusion with tissue fixative (GenoStaff, Tokyo, Japan) and the whole brain was embedded in paraffin. A 434-bp DNA fragment corresponding to the nucleotide position 154–587 of the rat *dsm-1* cDNA was subcloned into the pBluescript II KS(+) vector (Stratagene, La Jolla, CA, USA). DIG-labelled single-stranded riboprobes (probe 2, antisense and sense as the control) were prepared by *in vitro* transcription using the T7 or T3 RNA polymerase (DIG northern starter kit, Roche, Nonnenberg, Germany). Hybridization was performed with the DIG-labelled RNA probes at 60°C for 18 h, then samples were incubated with the anti-DIG AP conjugate (Roche). The colour reactions were performed with BCIP and NBT. The tissues were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan).

Semi-quantitative RT-PCR

The total RNA was extracted from tissues using an RNA extraction kit (RNeasy Midi Kit, Qiagen Inc., Valencia, CA, USA) and the complementary DNA (cDNA) synthesis was carried out using a SuperScript First Strand Synthesis System (Invitrogen) according to the manufacture's protocol. The PCR parameters used were: *dsm-1*, 94°C for 30 s, 68°C for 1 min, 72°C for 1 min with five cycles, and 94°C for 30 s, 65°C for 45 s, 72°C for 1 min with 31 cycles; GFAP, 94°C for 2 min, 60°C for 1 min, 72°C for 1 min for the first cycle, and 94°C for 30 s, 60°C for 45 s, 72°C for 1 min with 26 cycles, and; GAPDH, 94°C for 30 s, 55°C for 45 s, 72°C for 2 min with 25 cycles. The following primers were used: *dsm-1*, 5′-TCCCCGGACAGAGACAGCAGAATC-3′ (339–372) and 5′-GTGAAGACGGCGGCCCCAAACT-3′ (1137–1158); GFAP, 5′-CCCGGCTGGAGGTG-3′ (418–436) and 5′-CAGTTGGCGGC-GATAGTCATTAGC-3′ (863–886); GAPDH, 5′-TGCTGAGTATG-TCGTGGAGTCT-3′ (1116–1137) and 5′-AATGGGAGTTGCTGT-TGAAGTC-3′ (1696–1717).

Expression and functional assay of *dsm-1* in *Xenopus* oocyte

The open reading frame of *dsm-1* cDNA was subcloned into the pBScMxt vector, which contains the 5′- and 3′ untranslated regions of *Xenopus* β-globin gene for stable expression of cRNA in the oocytes (Ugawa *et al.* 2001). The cRNA was synthesized by *in vitro* transcription using T3 RNA polymerase (mMESSAGE mMA-CHINE, Ambion) after the linearization of the plasmid by *SalI* digestion. To examine the ion dependency for uptake of D-serine, the oocytes were incubated in standard FR uptake solution, Na^+-free FR solution (sodium was displaced by choline), and Cl^--free FR solution (chloride was displaced by gluconate).

Efflux measurements

Oocytes were pre-loaded with 1 mM of [^{3}H]D-serine for 60 min at 26°C. After non-specific binding of the radiolabelled compound on the cell surface was removed by brief wash with FR solution at room temperature (22–26°C), 10 groups of six oocytes were transferred to the D-serine-free FR solution and incubated for 30–120 min at 26°C. Then, the radioactivity in the medium and that remaining in the oocytes were measured. The rate of efflux was calculated as the percentage of radioactivity in the medium to the total pre-loaded radioactivity [radioactivity in medium/(radioactivity in medium + radioactivity remaining in oocytes) × 100%] (Fukasawa *et al.* 2000).

HPLC analysis of amino acids

In some experiments, the accumulation of unlabelled D-serine and other amino acids in the oocytes were evaluated: the simultaneous determination of the amino acid enantiomers and non-chiral amino acids was accomplished by HPLC with fluorometric detection as previously described (Hashimoto *et al.* 1992a; Sakurai *et al.* 2004). In brief, the 3–5 oocyte sample was sonicated in 10 volumes of 4% trichloroacetic acid after the addition of D-homocysteic acid as an internal standard, and the sample was centrifuged at 14 500 *g* for 20 min at 4°C. The supernatant was stored at −80°C until derivatization. The resulting sample was derivatized with N-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) and *o*-phthaldialdehyde for 2 min at room temperature and immediately applied to the HPLC system.

Cell culture

The type-1 astrocytes cultures were obtained by a standard shaking method (McCarthy and de Vellis 1980). The mixed glial culture was prepared from the Wistar rat cerebral cortex at post-natal day 1–2. After reaching subconfluence, the cultures were shaken at 80 r.p.m. for 10 min on the incubator shaker (EMMS-510; EYELA Tokyo Rikakikai Co., Tokyo, Japan) to deplete the microglia. After washing and recovery in a CO_2 incubator, the cultures were further shaken overnight at 250 r.p.m. to dislodge the population of type-2 astrocytes from the underlying layer of type-1 astrocyte. For the induction of the neurite outgrowth, PC12 cells were incubated in the medium supplemented with nerve growth factor (NGF) (100 ng/mL, Invitrogen) for 48 h.

Transfection of COS-7 cells and immunofluorescence microscopy

Using the Gateway cloning system (Invitrogen), the open reading frame of *dsm-1* cDNA was subcloned into pcDNA6.2/cLumio-DEST containing two epitope tag sequences (Lumio and V5) at the C terminus of the Dsm-1 protein in frame. The COS-7 cells were transfected with pcDsm-1/cLumio-DEST, in which *dsm-1* was inserted, or pcDNA6.2/cLumio-DEST as the control, by lipofection (Lipofectin, Invitrogen) according to the manufacturer's protocol. After 48 h, the transfected cells were stained with Lumio Green reagent (Lumio In-Cell Labelling kit, Invitrogen) without fixation according to the instruction manual. Alternatively, the cells were

fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature.

Because the protein products of the human orthologue of the isolated clone, *dsm-1*, have been reported to show a subcellular localization in Golgi membrane, we examined the possible co-localization of the V5-tagged Dsm-1 and a typical marker for the Golgi, mannosidase II (Rowe *et al.* 1999), using a double immunostaining technique. The cells were then stained with an anti-V5 monoclonal antibody and rabbit anti-mannosidase II polyclonal antibody (Chemicon, Temecula, CA, USA) for 60 min at room temperature, washed four times with PBS, and incubated with the Alexa Fluor 488-conjugated anti-mouse IgG antibody and the Alexa Fluor 594-conjugated anti-rabbit IgG antibody for 60 min at room temperature.

Data analysis and statistical comparison

The kinetic parameters of the D-serine uptake were calculated by the non-linear least-squares curve fitting method (GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA) and verified by the Lineweaver–Burke double reciprocal plot based on the Michaelis–Menten equation. The results were expressed as means with SEM. The statistical significance of the data was evaluated by the Student's *t*-test, or a one-way analysis of variance (ANOVA) followed by the Sheffe post-hoc test.

Results

Functional cloning of *dsm-1* cDNA from rat cerebral neocortex

Using the *Xenopus* oocyte expression screening of the rat cerebral neocortex cDNA library for the modulators of the D-serine accumulation, we have isolated a cDNA clone, designated as *dsm-1* (D-serine modulator-1), encoding the Dsm-1 protein that reduced the accumulation of D-serine into the cells when expressed in the oocytes. The *dsm-1* cDNA consisted of 1997 bp with the open reading frame (from nucleotide 56–1348) encoding a hydrophobic protein of 431 amino acids with the predicted mass of 47.3 kDa (Fig. 1a). The initiating ATG and surrounding sequence satisfied Kozak's translational initiation consensus (Kozak 1991). The cDNA possessed the typical polyadenylation signal (AATAAA) at the 20 nucleotide upstream of the poly(A) stretch.

According to the Kyte–Doolittle hydropathy analysis (Kyte and Doolittle 1982), the Dsm-1 protein possessed multiple hydorophobic regions throughout the sequence (Fig. 1b). Thus, it consisted of seven prominent hydrophobic domains (hydrophobic domains 1–7) and a long hydrophobic stretch (residue 340–410, hydrophobic domains 8–10). The potential protein kinase A and protein kinase C phosphorylation sites were located at residues 100, 231, 234, 263 and 335. The potential casein kinase II phosphorylation sites were found at residues 144 and 257. There was no N-linked glycosylation consensus site. The C-terminal region containing the RXXK and PXXP motifs at residues 417–426 was predicted to be recognized as a class I ligand of the SH3 domain (Feng *et al.* 1994; Lewitzky *et al.* 2004). The long hydrophobic stretch near the C-terminus was reported to be characteristic of neutral amino acid transporters ASCT1, ASCT2, and glutamate transporters (Arriza *et al.* 1993; Shafqat *et al.* 1993; Utsunomiya-Tate *et al.* 1996). However, Dsm-1 did not have a sequence similar to any of these transporters.

Based on the BLAST search, we found that Dsm-1 was highly homologous to a transporter for the 3′-phsophoadenosine 5′-phosphosulfate (PAPS) recently isolated in human and *Drosophila* (Kamiyama *et al.* 2003; Luders *et al.* 2003). A multiple alignment analysis revealed that the Dsm-1 protein possessed 91.8 and 43.8% amino acid identities to the human PAPST1 and *Drosophilia* SLL proteins, respectively (Fig. 1c). Based on these results, we concluded that *dsm-1* was the rat orthologue of the human *PAPST1* gene.

Detection of the *dsm-1* gene and its transcript

In Southern blotting using 1.3-kb of a ^{32}P-labelled probe (probe 1) for *dsm-1*, we detected a single band at 4.1 kb, 3.9 kb and 4.2 kb in the rat genomic DNA digested with *Hin*dIII, *Bam*HI and *Eco*RI, respectively (Fig. 2a). This indicates that the *dsm-1* gene exists as a single copy in the rat genome.

In the northern blot analysis using the same probe, a transcript at 2.1 kb was detected with 10 μg of the total RNA from rat cerebral neocortex (Fig. 2b). The size of this transcript is consistent with that of *dsm-1* cDNA extended by 5′ rapid amplification of the cDNA ends (RACE)-PCR, suggesting that we obtained a full-length cDNA for *dsm-1*. With a shorter non-RI (radio isotope) riboprobe (434 bases, probe 2) that also detected a single 2.1-kb transcript in the neocortical mRNA preparation (Fig. 2b), we further examined the distribution of the *dsm-1* transcript in the various organs (Fig. 2b) and within the central nervous tissues (Fig. 2c). The *dsm-1* mRNA was predominantly expressed in the liver and brain, slightly in the kidney and heart, and at the trace or non-detectable levels in the testis, skeletal muscles, lung and spleen (Fig. 2b). The central nervous tissues showed an uneven distribution of the *dsm-1* transcript, that the signal intensity of the northern blotting was higher in the hippocampus, olfactory bulb, thalamus and cerebral cortex, much lower in the spinal cord, hypothalamus, and midbrain, and faint in the cerebellum and pons-medulla (Fig. 2c).

Similarly, *in situ* hybridization analysis with the same antisense probe (probe 2) revealed that a well-contrasted hybridization signal was observed throughout the brain tissues with a particularly high density in the olfactory bulb, layers II-IV of the cerebral neocortex, pyramidal layers of the CA1 and CA3 regions and dentate gyrus in the hippocampus, and medial habenular nucleus (Figs 3a, c and e). No significant mRNA signal was detected with the corresponding sense probe (Figs 3b, d and f).

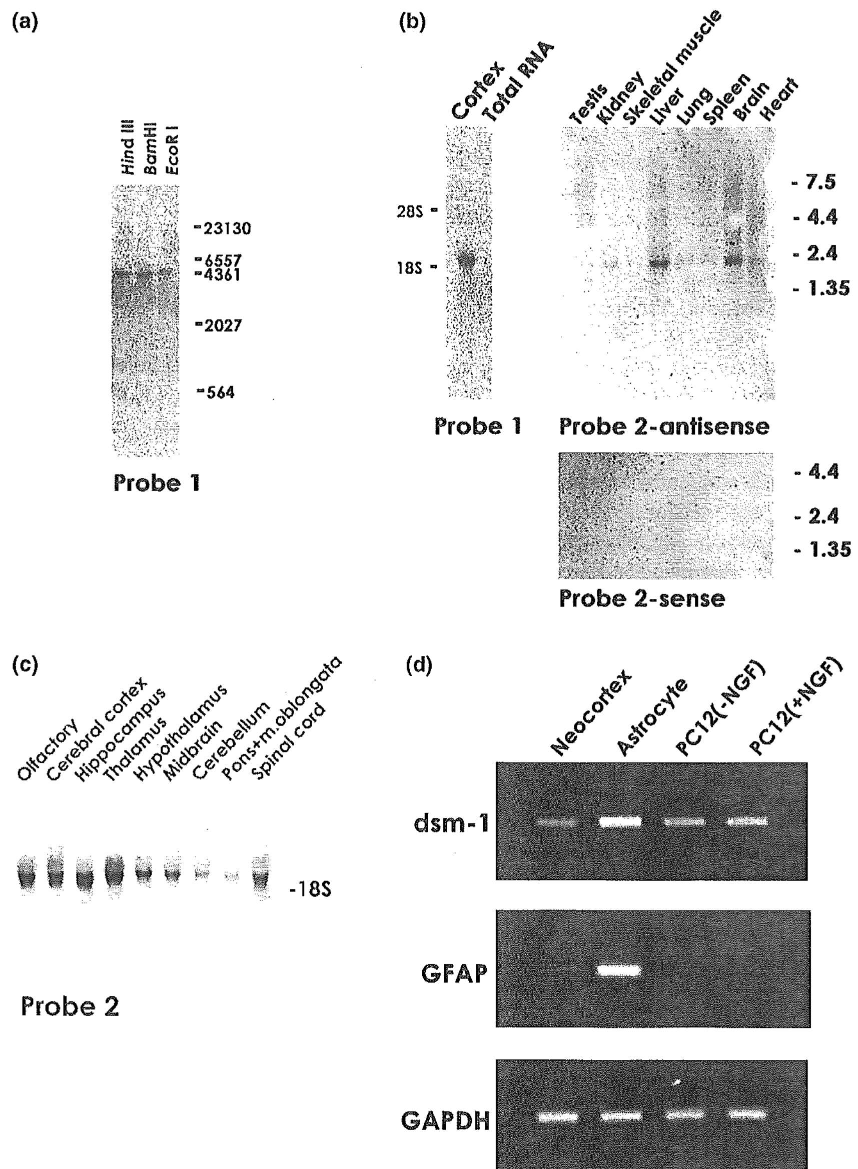

Fig. 2 Southern and northern blot, and RT-PCR analyses of *dsm-1*. (a) Southern blotting of rat genomic DNA. With a 1.3-kb probe (probe 1, see Materials and methods) for *dsm-1*, a single band at 4.1, 3.9 and 4.2 kb is detected in the rat genomic DNA digested with *Hin*dIII, *Bam*HI and *Eco*RI, respectively. (b, c) Northern blotting. With the same probe, the expression of *dsm-1* mRNA was detected at 2.1 kb in the total RNA from the neocortex of the adult rats (b, left panel). Using a shorter probe (probe 2, antisense strand), the signal of the same size was detected in a multiple tissue blot (b, right panel) and a brain tissue blot (c). This probe was used for the *in situ* hybridization (Fig. 3). (d) Predominant expression of *dsm-1* in primary cultured astrocytes. The expression of *dsm-1* mRNA was determined by RT-PCR in different cells in culture: primary cultured astrocytes, undifferentiated PC12 cells (–NGF), and PC12 cells with neuronal differentiation (+NGF). The reliabilities of the cell preparation and RT-PCR were examined by the detection of the GFAP and GAPDH mRNAs.

We further found a substantial expression of *dsm-1* mRNA in the primary cultured astrocytes and the PC12 cells treated with or without NGF by RT-PCR (Fig. 2d). The astrocytes seemed to express a higher level of *dsm-1* than the undifferentiated (–NGF) or neuronally differentiated (+NGF) PC12 cells (Fig. 2d).

Effects of Dsm-1 expression on [^{3}H]D-serine accumulation in the *Xenopus* oocytes

To clarify the detailed effects of the Dsm-1 protein on cellular D-serine accumulation, the uptake assay of D-serine was conducted in the *Xenopus* oocyte expression system. We verified using RT-PCR that the sufficient levels of *dsm-1* cRNA remained detectable in the oocyte for at least 2 days after microinjection of the cRNA, but not of H_2O alone (Fig. 4a). This verification rejected the possible expression of endogenous *dsm-1* in the assay system.

The amount of accumulated [^{3}H]D-serine in the cells for 1 h at 4°C was below 5% of that at 26°C. This temperature-dependent nature suggests that the accumulation of D-serine is mainly because of an energy-dependent transportation into the cells.

As shown in Fig. 4(b), the microinjection of the sense strand of *dsm-1* cRNA, but not of its antisense strand or H_2O, reduced the accumulation of [^{3}H]D-serine into the oocytes by approximately 28%. This inhibitory effect of *dsm-1* on the D-serine accumulation depended upon the dose of the injected *dsm-1* cRNA (Fig. 4c). The intrinsic D-serine accumulation in the *Xenopus* oocyte was found to be diminished in the absence of sodium [Na^+(–), 67% of

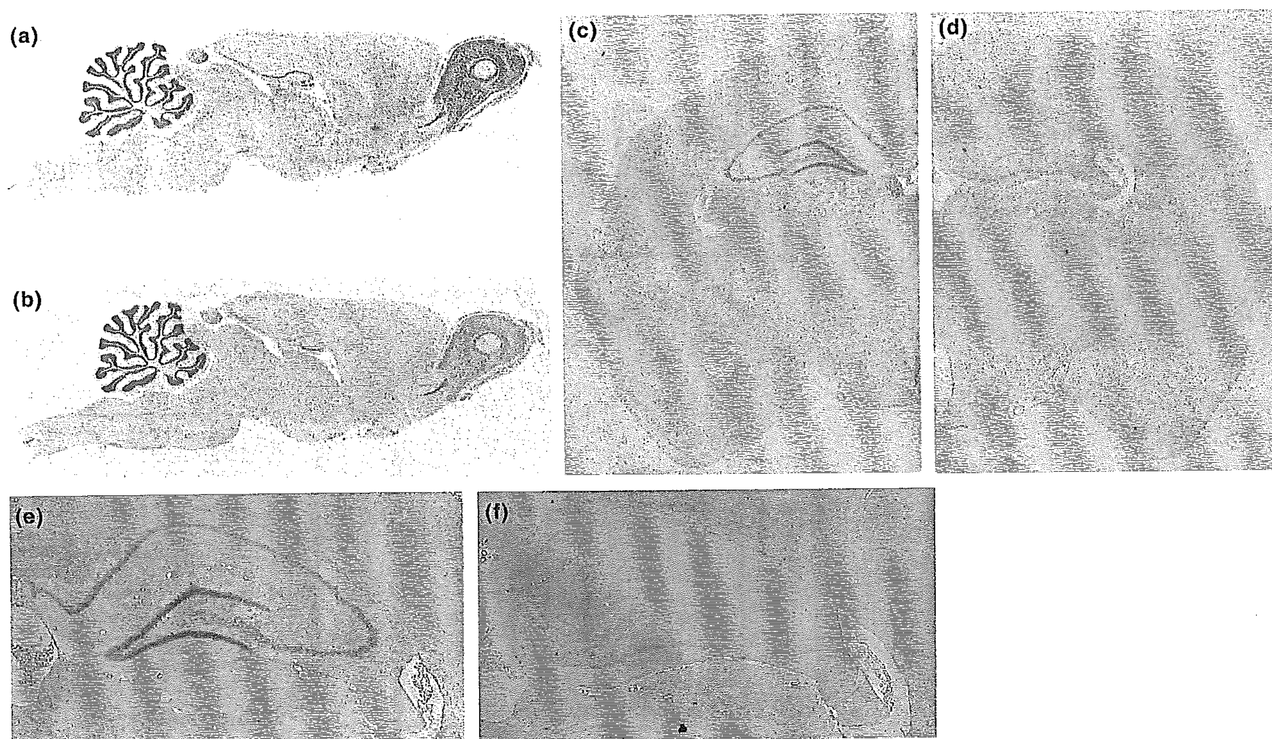

Fig. 3 *In situ* hybridization histochemistry of *dsm-1* in rat brain. The sagittal section (a, b) and the coronal section at hippocampus level (bregma −3.30 mm) (c–f) of the rat brain were hybridized with the *dsm-1* antisense (a, c, e) and sense (b, d, f) strands of probe 2. The RNA signals are detected by the DIG system as shown in the purple colour, and the cell nuclei are counterstained by Kernechtrot solution as shown in pink. Note that the *dsm-1* mRNA was detectable throughout the central nervous system with a concentration in the olfactory bulb, cerebral neocortex, hippocampus and medial habenula. No signal was observed with the corresponding sense probe (b, d, f).

control] and chloride [Cl^-(–), 50%] ion (Fig. 4d). These data suggest that D-serine could be taken up into the cells through sodium and chloride ion-dependent and -independent transporters. The *dsm-1* cRNA injection apparently attenuated all of these accumulation processes (Fig. 4d).

The decreasing effects of the *dsm-1* expression on the D-serine accumulation in the presence of sodium and chloride ion observed at the extracellular D-serine concentrations of 10 and 100 μM (Fig. 4e) therefore could play a physiological role in the mammalian D-serine-rich brain regions that exhibit relatively high contents of extracellular D-serine around 10 μM (Hashimoto *et al.* 1995a; Matsui *et al.* 1995). Figure 4(f) illustrates a velocity–substrate concentration plot of unlabelled D-serine accumulation into the oocyte at concentrations ranging from 10 to 3000 μM and a Lineweaver–Burk plot of these data. Non-linear least squares model fitting of the data from the saturation experiments for the Michaelis–Menten equation indicated that the D-serine accumulation in the oocyte injected with H_2O and *dsm-1* cRNA occurred using a single-site model having apparent kinetic parameters which were: H_2O, K_m 65 ± 9 μM, V_{max} 1.50 ± 0.04 pmol/oocyte/h; dsm-1, K_m 40 ± 7, V_{max} 1.03 ± 0.04 ($n = 6$: Fig. 4f).

Effects of Dsm-1 expression on the basal contents and accumulation of various amino acids in the *Xenopus* oocytes

The present HPLC measures further revealed that the *dsm-1* cRNA injection into the *Xenopus* oocytes produced differential effects on the basal concentrations and accumulations of various amino acids in the cells. Thus, the expression of the Dsm-1 protein significantly reduced the contents of the intrinsic D-serine (−62%), L-serine (−46%), L-threonine (−35%) and glycine (−14%) and increased those of the cellular L-glutamine (+57%) and L-aspartate (+18%) when the oocytes were incubated in the medium without any amino acid (Table 1). As shown in Fig. 5(a), the intraoocyte accumulation of D-serine in the presence of extracellular D-serine (100 μM) was attenuated by the *dsm-1* expression. In contrast, the *dsm-1* expression failed to affect the taurine accumulation following extracellular loading of taurine at 100 μM. Moreover, during the incubation in the medium containing various amino acids at each concentration of 100 μM, the *dsm-1* cRNA injection significantly diminished the accumulation levels of D-serine (−29%), L-serine (−11%) and L-threonine (−10%), and augmented those of L-glutamine (+54%) and L-glutamate (+32%) without any

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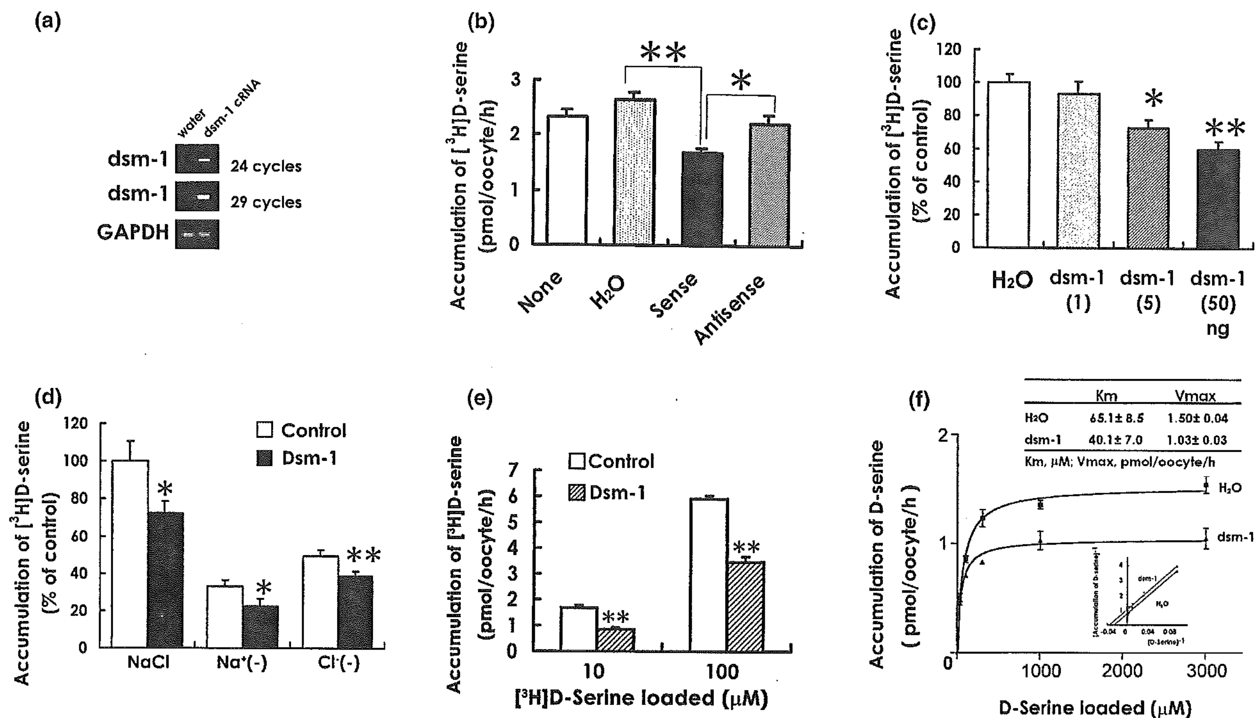

Fig. 4 Modification by Dsm-1 expression of [3H]D-serine accumulation in the *Xenopus* oocytes. (a) Detection of *dsm-1* in *Xenopus* oocytes after the injection of cRNA. RT-PCR was performed to confirm that the *dsm-1* transcript was detectable at the time point of the D-serine uptake assay 2 days after the microinjection of *dsm-1* cRNA. The intrinsic expression of *dsm-1* was not detectable in the *Xenopus* oocytes injected with H_2O alone. (b) Modification of cellular accumulation of D-serine by *dsm-1*. The accumulation of [3H]D-serine was measured at 10 μM in the *Xenopus* oocytes without injection (None), or injected with water (H_2O), *dsm-1* sense strand (Sense) and antisense strand (Antisense) cRNA. The expression of *dsm-1* resulted in a significantly decreased accumulation of [3H]D-serine to the oocytes, while there was no significant difference in the oocytes injected with water and the *dsm-1* antisense cRNA. $*p < 0.05$, $**p < 0.01$ between the experimental groups linked by the respective solid lines. (c) cRNA dose-dependent effect of *dsm-1*. The accumulation of [3H]D-serine was measured in the oocytes injected with 1, 5 and 50 ng of *dsm-1* cRNA. The accumulation of D-serine was significantly decreased in accordance with the increasing expression of *dsm-1*. $*p < 0.05$, $**p < 0.01$ versus water-injected controls. (d) Ion-dependency of the inhibitory effect of Dsm-1. The accumulation of [3H]D-serine (10 μM) was measured in Frog Ringer as authentic uptake solution (labelled as NaCl), or in the uptake solutions in which the Na-ion was displaced by choline [Na^+(–)], or Cl-ion was displaced by gluconate [Cl^-(–)]. $*p < 0.05$, $**p < 0.01$ as compared with respective water-injected control. (e) Effect of Dsm-1 at various concentrations of extracellular D-serine. The [3H]D-serine accumulation in the oocytes expressing *dsm-1* was measured at 10 and 100 μM loaded. $*p < 0.05$, $**p < 0.01$ as compared with respective water-injected control. (f) Effect of *dsm-1* on the kinetics of the D-serine uptake activity in the *Xenopus* oocytes. The accumulation of unlabelled D-serine in the oocytes expressing *dsm-1* was assayed and measured by HPLC. The kinetics of the accumulation of D-serine loaded from 10 to 3000 μM was calculated by a non-linear regression curve fitting method. Inset, the Lineweaver–Burke double reciprocal plot of D-serine accumulation. The values are expressed as means ± SEM.

effects on the accumulations of the other amino acids studied (Fig. 5b).

Effects of Dsm-1 expression on the rate of [3H]D-serine efflux from the *Xenopus* oocytes

To address the question of how the Dsm-1 reduces the basal contents and accumulation of D-serine in the oocytes, we studied the influences of Dsm-1 expression on the rate of [3H]D-serine efflux that was calculated as the percentage of the total pre-loaded [3H]D-serine radioactivity [radioactivity in medium/(radioactivity in medium + radioactivity remaining in oocytes) × 100%]. As shown in Fig. 6(a), the increased efflux rates for 30, 60 and 120 min were observed in the oocytes expressing *dsm-1* as compared with respective water-injected control oocytes. Furthermore, Dsm-1 expression, but not water injection, caused a significant and time-related decrease in the amount of the pre-loaded [3H]D-serine in the oocytes during the 120-min incubation in the D-serine-free medium (Fig. 6b). However, the decreasing effects of Dsm-1 disappeared under the condition of the incubation at 4°C (data not shown). These findings suggest that Dsm-1 might facilitate the temperature-dependent release of the pre-loaded [3H]D-serine from the oocytes.

Table 1 Effect of *dsm-1* for endogenous amino acids in *Xenopus* oocyte

Treatment	H_2O	*dsm-1* cRNA	
Amino Acid	pmol/oocyte	pmol/oocyte	% of control
Neutral amino acids			
Gly	21.3 ± 0.9	18.4 ± 0.9*	86.3
L-Ala	8.12 ± 1.21	7.61 ± 0.77	93.8
β-Ala	1.35 ± 0.07	1.23 ± 0.05	91.1
L-Ser	52.4 ± 3.9	28.5 ± 2.8**	54.4
D-Ser	0.225 ± 0.017	0.0850 ± 0.0086**	37.8
L-Thr	31.0 ± 2.0	20.2 ± 1.2**	65.0
L-Asn	16.0 ± 0.8	16.6 ± 0.5	103.7
L-Gln	14.6 ± 1.6	22.9 ± 1.6**	156.7
Tau	1.63 ± 0.11	1.66 ± 0.09	101.6
Acidic amino acids			
L-Asp	267 ± 12	316 ± 9**	118.4
L-Glu	302 ± 18	342 ± 19	113.2
Basic amino acids			
L-Arg	92.9 ± 4.4	81.1 ± 3.1	87.2

Endogenous amino acids in the oocytes expressing *dsm-1* were simultaneously measured by HPLC in the absence of any amino acids in the medium. The results represent the mean with SEM of the date obtained from 10 groups of three oocytes. $*p < 0.05$, $**p < 0.01$ as compared with respective water-injected controls (*t*-test).

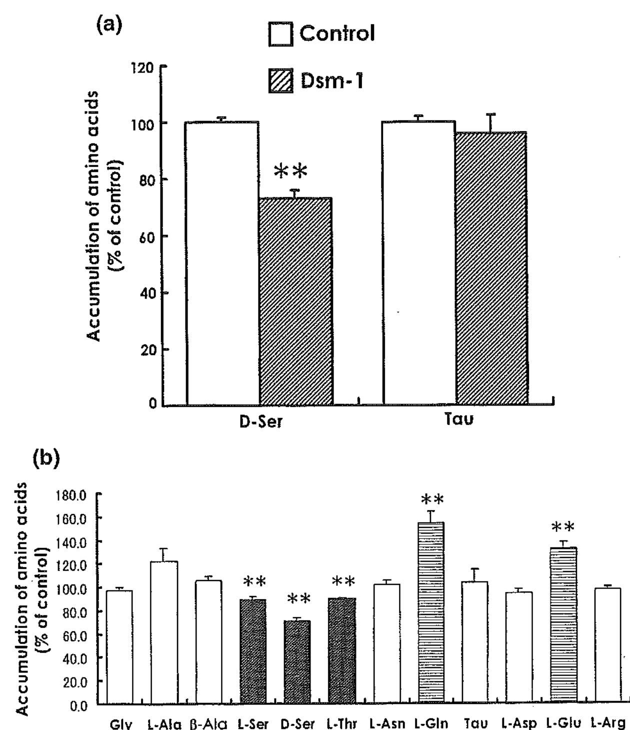

Fig. 5 Effect of Dsm-1 expression on the accumulation of various amino acids in the *Xenopus* oocytes. A, Effect of Dsm-1 on the accumulation of D-serine and taurine. The accumulation of D-serine and taurine, individually loaded at 100 μM, were, respectively, measured by HPLC. The oocytes expressing *dsm-1* reduced the accumulation of D-serine but did not alter the accumulation of taurine in the oocytes. The respective control values were (pmol/oocyte/h): D-serine 0.734 ± 0.023, tau 2.06 ± 0.08. $*p < 0.05$, $**p < 0.01$ as compared with the water-injected control. (b) Effect of Dsm-1 on the accumulation of various amino acids loaded. The accumulation of each amino acid in the oocytes was simultaneously measured by HPLC in the presence of 100 μM of various amino acids (gly, L-ala, β-ala, L-ser, D-ser, L-thr, L-asn, L-gln, tau, L-asp, L-glu and L-arg). The results are expressed as a percentage of the control values of the respective amino acids. The Dsm-1 significantly reduced the accumulation of the D-serine, L-serine and L-threonine to the oocytes. The respective control values were (pmol/oocyte/h): gly 19.7 ± 0.5, L-ala 5.20 ± 0.53, β-ala 1.13 ± 0.02, L-ser 59.2 ± 1.4, D-ser 0.351 ± 0.012, L-thr 23.0 ± 0.6, L-asn 29.2 ± 0.9, L-gln 22.4 ± 0.9, tau 1.76 ± 0.07, L-asp 263 ± 5, L-glu 250 ± 9, L-arg 138 ± 4. $*p < 0.05$, $**p < 0.01$ as compared with the water-injected control.

Subcellular localization of Dsm-1 protein

The subcellular localization of the tagged Dsm-1 fusion protein was examined in COS-7 cells transfected with the Lumio-V5-tagged fusion protein construct. In the fixed COS-7 cells, the immunoreactivity of the V5-tagged Dsm-1 protein exhibited a punctuate distribution throughout the cytoplasm and was asymmetrically concentrated near the nucleus (Fig. 7a). Moreover, in the perinuclear portions (Fig. 7c), the immunoreactivity of the tagged Dsm-1 was found to be co-localized with that of a specific marker for the Golgi, mannosidase II, when the cells were double immunostained with an anti-V5 monoclonal antibody and an anti-mannosidase II polyclonal antibody. This indicates that Dsm-1 protein may be enriched at least in the Golgi apparatus. We did not observe any significant immunoreactivity in the cells transfected with the vector alone (Fig. 7d). The result that a similar patchy distribution pattern of the Lumio fluorescence was detected in the unfixed cells transfected with the above mentioned *dsm-1* construct (Fig. 7g) denies the possibility that the Dsm-1 fusion protein-like immunoreactivity to the anti-V5 antibody may be an artifact signal as a result of the cell fixation and/or permeabilization processes.

Discussion

We have presently isolated by screening the neocortical cDNA library using a functional cloning technique, a novel rat transcript, designated as *dsm-1* (D-serine modulator-1), that reduces the accumulation of D-serine in the gene-expressing *Xenopus* oocyte. *dsm-1* has been predicted to be the rat orthologue of the human 3′-phsophoadenosine 5′-phosphosulfate transporter 1 (*PAPST1*) gene (Kamiyama *et al.* 2003). We have observed significant *dsm-1* mRNA signals in the D-serine-rich forebrain regions, and the patchy cytoplasmic and Golgi apparatus-related distribution of the peptide-tagged Dsm-1 fusion protein transiently expressed in the COS-7 cells. These findings indicate that *dsm-1* may, at

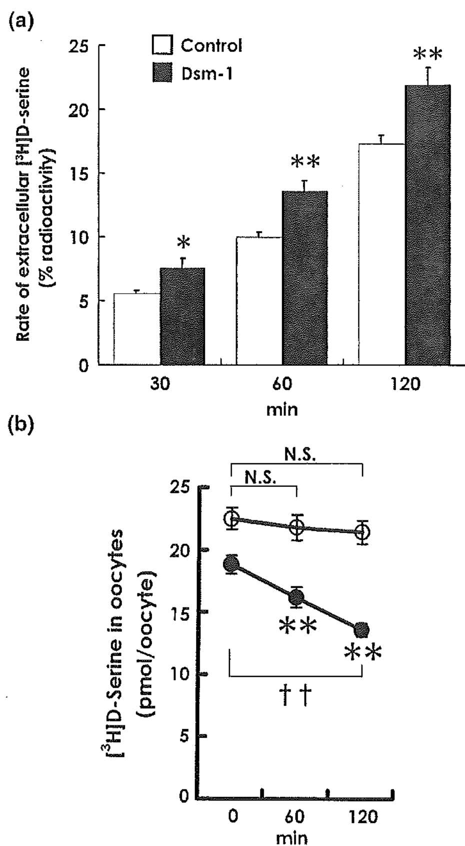

Fig. 6 Effect of Dsm-1 expression on the rate of [^{3}H]D-serine efflux from the *Xenopus* oocytes. Oocytes expressing *dsm-1* (black column and circles) and control oocytes injected with water (open column and circles) were pre-loaded with [^{3}H]D-serine for 60 min. The efflux of the pre-loaded [^{3}H]D-serine into the FR solution (a) and the radioactivity remaining in the oocytes (b) were measured. The rate of efflux was calculated as the percentages the radioactivity in the medium to the total pre-loaded radioactivity [radioactivity in medium/(radioactivity in medium + radioactivity remaining in oocytes) × 100%] (a). *$p < 0.05$, **$p < 0.01$ as compared with the water-injected control. ††$p < 0.01$ as compared with the D-serine pre-loaded control.

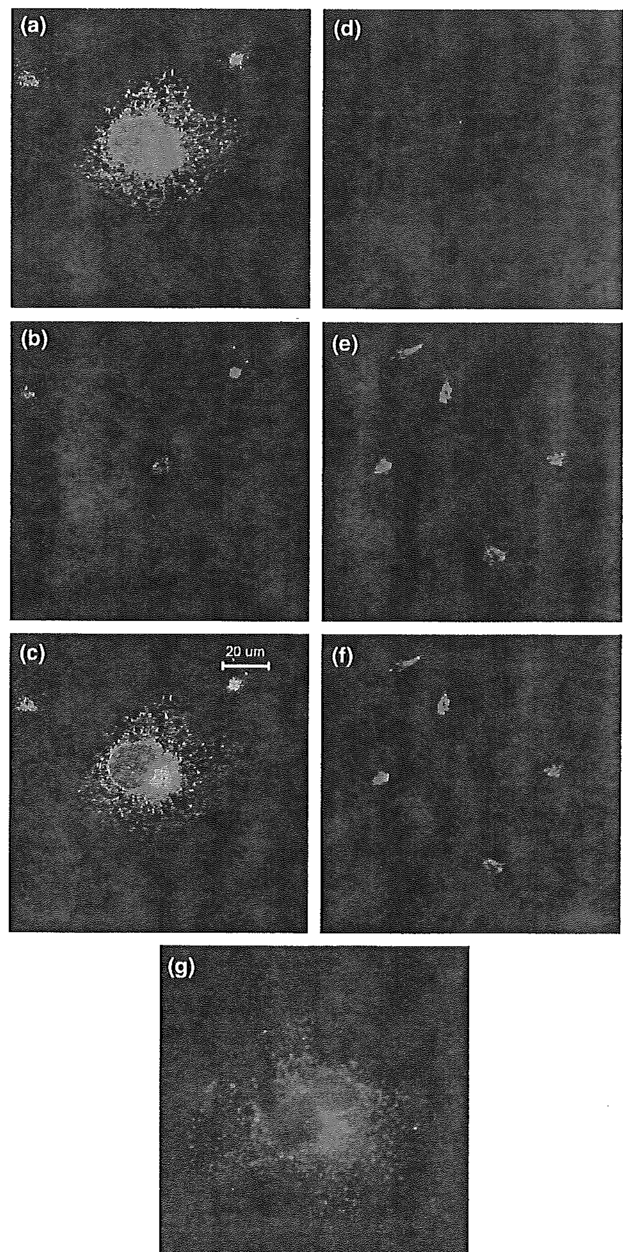

Fig. 7 Subcellular localization of Dsm-1 protein expressed in COS-7 cells. COS-7 cells were transfected with the Lumio-V5-tagged *dsm-1* fusion construct (a, b, c, g) or vector alone as a control (d, e, f). The Dsm-1 fusion protein was observed by the immunofluorescence detection with the anti-V5 monoclonal antibody (a–f) after the fixation, or by fluorescent detection of unfixed cells with the Lumio reagent (g). Double staining was performed for V5 tag and mannosidase II (a–f). The image of V5 and mannosidase II were merged (c, f). The Dsm-1 fusion protein demonstrates a punctuate distribution throughout the cell with a concentration near the nucleus (a, c), while no significant signal is observed in the cell transfected with the vector alone (d).

least in part, be involved in the D-serine translocation across the vesicular or plasma membranes in the rat brain.

As expected by the 92% homology of the predicted amino acid sequence between rat Dsm-1 and the human PAPST1 protein, the deduced Dsm-1 protein has been shown to have 10 transmembrane domains (Figs 1b and c) like human PAPST1. PAPST1 has been reported to exhibit the Golgi apparatus-preferring distribution in the SW480 cells (Kamiyama *et al.* 2003) and to translocate PAPS from cytosol into the Golgi lumen where sulfation by PAPS as the sulfate donor of proteoglycans, secretory and membrane proteins and glycolipids occurs. Mutations in the *PAPST1* gene have been demonstrated to result in the lack of sulfation of glycosaminoglycans by the sulfotransferase. Therefore, rat Dsm-1 may carry PAPS and participate in the above sulfation processes in the cell organelles of the rat. This idea seems to be supported by the PAPST1-like subcellular distribution of the tagged Dsm-1 protein in the COS-7 cells.

Hydropathy analysis has revealed that Dsm-1 contains a characteristic long hydrophobic stretch situated in the C-terminal half of the protein (hydorophobic domains 8–10 in Fig. 1b) -like glutamate transporters and neutral amino acid transporters including ASCT-1 (Arriza *et al.* 1993; Shafqat *et al.* 1993), ASCT-2 (Utsunomiya-Tate *et al.* 1996). This structural profile suggests that *dsm-1* may also act as a transporter carrying glutamate and/or some neutral amino acids in the brain. This speculation is consonant with the fact that the *dsm-1* expression causes an alteration in the basal contents and/or accumulation of D-serine, L-serine, L-threonine, L-glutamate, L-glutamine and glycine, but not of the other amino acids tested including L-aspartate, L-asparagine, L-alanine, β-alanine, taurine and L-arginine, in the *Xenopus* oocyte (Table 1 and Fig. 5b).

The mRNAs of *dsm-1* are found to be predominantly expressed in the brain and liver. However, the expression of the human homologue of *dsm-1*, *PAPST1*, has been reported in these organs, but to be relatively higher in the skeletal muscle and testis where *dsm-1* is only slightly transcribed in the rat. These discrepancies may be caused by the distinct regulations of the *PAPST1* genes and/or could reflect the differences in their physiological functions between rats and humans. Interestingly, the present *in situ* hybridization study is the first to reveal that, in the rat brain, *dsm-1* mRNA is widely distributed with a forebrain-dominant gradient to the hindbrain. The observation that cultured astrocytes display a more intense *dsm-1* expression than the whole neocortex and differentiated PC12 cells to neurons indicates that *dsm-1* and its protein products might be enriched in the glial cells in the neural tissues.

Together with the differential influences of the *dsm-1* expression on various amino acids, the uneven distribution of its mRNA among the brain regions and neuronal cell types raises the possibility that Dsm-1 could interact with some of the specific amino acid systems in the brain, although the transporter-like amino acid sequence of the C-terminal half region is not identical to those of any known amino acid transporters.

The *Xenopus* oocyte used for the screening of the transcripts encoding D-serine carriers in this study seems to express no *dsm-1* transcript (Fig. 4a), but to possess intrinsic D-serine and a plasma membrane system that is capable of transporting the D-amino acid, because we detected a trace level of D-serine in the non-treated oocytes and an increase in the D-serine contents when the oocytes were incubated in the medium containing D-serine. The presence of D-serine in the *Xenopus* oocyte is not surprising based on the previous report showing that very low contents of D-serine are detected in the brain of the bullfrog (*Rana catesbeiana*) (Nagata *et al.* 1994) while the D-serine amount has never been determined in the peripheral tissues of amphibians.

The possibility that the down-regulation by *dsm-1* of the basal levels and the accumulation of D-serine in the oocytes may be because of an artifact or a non-specific phenomenon is excluded by the following results of the present oocyte experiments: (i) no changes in the basal contents and accumulation of D-serine after water and *dsm-1* antisense cRNA injection (Fig. 4b), (ii) the cRNA dose-related nature of the effects of the *dsm-1* expression on the D-serine accumulation (Fig. 4c), (iii) differential effects of the *dsm-1* expression on the basal contents and accumulation of various amino acids (Table 1 and Fig. 5b), and (iv) lack of influence by the *dsm-1* expression on the taurine contents in the oocytes with and without loading of the extracellular taurine (Table 1 and Fig. 5a).

It is more likely that the Dsm-1-induced changes in the intracellular levels of D-serine could be associated with the release process of the D-amino acid, because the expression of *dsm-1* protein products has been shown to accelerate the rate of efflux of the pre-loaded D-serine in the oocytes (Figs 6a and b). The accelerating effects and the transporter-like structure of Dsm-1 suggest that Dsm-1 may facilitate the temperature-dependent release of the pre-loaded [^{3}H]D-serine from the oocytes by pumping out the intracellular D-serine at the plasma membrane or by transporting the D-amino acid into some vesicles that could liberate the D-serine to the extracellular space after their fusion with the plasma membrane. The cytoplasmic and Golgi apparatus-, but not plasma membrane-, related subcellular distribution pattern of Dsm-1 seems to support the latter possibility. Dsm-1 might be expressed in the vesicular membrane and translocate the cytosolic D-serine taken up from the medium to some vesicles as a vesicular transporter. The transported D-serine then might be secreted through the fusion of these vesicles with the plasma membrane. It cannot be totally ruled out that Dsm-1 could transport D-serine to the lysosome or peroxisome to undergo the degradation process that may eliminate the intracellular D-serine. Dual immunohistochemical visualization of D-serine and Dsm-1 in the mammalian cells will help to clarify the relationship between the intracellular dynamics of D-serine and Dsm-1.

In support of the above assumption, the possible direct interaction of Dsm-1 with the sodium-dependent and -independent plasma membrane transporters that are capable of taking up D-serine (see Fig. 4d) appears to be negated by the observations that (i) the *dsm-1* products caused a decrease in the basal D-serine contents even in the absence of extracellular D-serine (Table 1), and (ii) an uncompetitive, but not competitive and non-competitive, inhibition was estimated in the *dsm-1*-induced reduction in D-serine accumulation by kinetic analysis using the Michaelis–Menten equation (Fig. 4f).

Because the inhibitory modification of cellular D-serine contents is observed at the medium D-serine content of 10 μM, which is close to the extracellular concentration of D-serine in the forebrain areas of the freely moving rodents, it is conceivable that *dsm-1* and its protein product might play a

physiological role in the regulation of the metabolism and functions of the endogenous D-serine in mammalian tissues. In fact, an intense constitutional expression of *dsm-1* mRNA is found in the D-serine- and NMDA receptor-rich brain regions such as the neocortex, hippocampus, and striatum (Hashimoto *et al.* 1993a; Schell *et al.* 1995). In the hippocampus, the hybridization signals of *dsm-1* mRNA occur throughout the tissue with a higher density in the pyramidal and granule cell layers, while a D-serine-like immunoreactivity is shown to be very weak or absent in these layers and concentrates in the glia of the molecular layers (Schell *et al.* 1995). These data and the robust *dsm-1* expression in the astrocyte cultured from the neocortex indicate that *dsm-1* mRNA could co-localize with D-serine in, at least, a part of the astrocytes in the molecular layers which has been shown to liberate D-serine (Yang *et al.* 2003) and contain D-serine degrading enzymes, serine racemase/serine dehydratase (Strisovsky *et al.* 2003; Foltyn *et al.* 2005) and D-amino acid oxidase (Urai *et al.* 2002).

In conclusion, the present study has demonstrated that the rat brain expresses the orthologue of the human *PAPST1* gene, *dsm-1*, that modulates the temperature-dependent D-serine accumulation in the *Xenopus* oocyte. From the biochemical, neuroanatomical and cell biological profiles of *dsm-1* and its protein product, it is proposed that Dsm-1 could play a role in the control over the intra- and extracellular contents of an endogenous co-agonist for the NMDA receptor, D-serine, in the mammalian brains. Because the dysfunctions in the D-serine–NMDA receptor interaction have been considered to be involved in the pathophysiology of neuropsychiatric disorders, including a type of schizophrenia (Nishikawa *et al.* 1994; Kumashiro *et al.* 1995) and of spinocerebellar degeneration (Ogawa *et al.* 2003), *dsm-1* may be a useful clue not only for the molecular mechanisms of the D-serine metabolism, but also for the development of a novel pharmacotherapy modulating D-serine signalling for these disorders. Besides Dsm-1 and D-serine interaction, the biological meanings of the significant effects of Dsm-1 on the intracellular contents of L-glutamate, L-glutamine, L-serine, L-threonine, and glycine (Table 1 and Fig. 5b) require further elucidation.

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Cloning of a D-serine-regulated transcript *dsr-2* from rat cerebral neocortex

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Abstract

D-Serine is now considered to be an endogenous co-agonist of the NMDA receptor in mammalian brain. To obtain insight into the molecular mechanisms underlying D-serine metabolism and function, we explored transcripts that are responsive to D-serine in the neocortex of the 8-day-old infant rat by a differential cloning technique, RNA arbitrarily primed PCR. We isolated a novel D-serine inducible transcript, D-serine-responsive transcript-2 (*dsr-2*), that was exclusively expressed in the brain. Sequence analysis of the corresponding cDNAs to the transcript revealed that the *dsr-2* mRNA consists of 7199 nucleotides with an open reading frame encoding 111 amino acids. The *dsr-2* gene was located on the reverse strand within an intron of the neurexin-3α gene, mapped to rat chromosome 6q24-31. The regional distribution of the basal expression of *dsr-2* and its ontogenic changes in the brain closely correlated with those of free D-serine and of NMDA receptor R2B subunit mRNA, but were somewhat different from those of the neurexin-3α transcript. These findings suggest that *dsr-2* may be involved in D-serine metabolism and/or function, and in the interactions between D-serine, NMDA receptor and neurexin-3α, in mammalian brain.

Keywords: D-serine, *dsr-2*, L-serine, neurexin-3α, rat neocortex, RNA arbitrarily primed PCR.
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Mammalian brains contain free D-serine for life at exceptionally high concentrations throughout life, although free D-amino acids had long been assumed to be unnatural in mammalian tissues (Hashimoto *et al.* 1992; Nishikawa *et al.* 1994; Fujii 2002). By selectively stimulating the glycine site of the NMDA receptor, D-serine acts as a co-agonist for the glutamate receptor (Dannysz and Parsons 1998), which is required for physiological activation of the NMDA receptor. It is now widely accepted that its distribution pattern and developmental changes are closely correlated with those of the NMDA receptor in the brain (Hashimoto *et al.* 1993a, 1993c, 1995b; Nishikawa *et al.* 1994; Schell *et al.* 1995, 1997). Together with the therapeutic efficacy of D-serine on schizophrenic symptoms (Nishikawa *et al.* 1994; Javitt 2004) and cerebellar ataxia (Ogawa *et al.* 2003), these pharmacological and neuroanatomical relationships suggest that D-serine may be an endogenous co-agonist of the NMDA receptor and may play a pivotal role in the regulation of higher brain functions (Nishikawa *et al.* 1994). In support of this view, selective elimination of endogenous D-serine by D-amino acid oxidase (DAO) without alteration in the glycine content attenuates NMDA receptor functions such as long-term potentiation, a model of memory, in rat hippocampal slice preparations (Yang *et al.* 2003).

The cellular and extracellular concentrations of D-serine might undergo precise control by a specific molecular system. Neurochemical studies have so far indicated the processes of biosynthesis (Dunlop and Neidle 1997; Takahashi *et al.* 1997), release (Hashimoto *et al.* 1995a; Schell *et al.* 1995), uptake (Wako *et al.* 1995; Hayashi *et al.* 1997; Yamamoto *et al.* 2001; Javitt *et al.* 2002; Ribeiro *et al.*

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Abbreviations used: DAO, D-amino acid oxidase; *dsr-2*, D-serine-responsive transcript-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; nt, nucleotides; PD, postnatal day; nrxn3α, neurexin-3α; ORF, open freading frame; RACE, rapid amplification of cDNA ends; RAP–PCR, RNA arbitrarily primed polymerase chain reaction.

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2002) and degradation (Hashimoto *et al.* 1993b; Urai *et al.* 2002) of endogenous D-serine in the brain. The putative serine racemase that synthesizes D-serine from the L-enantiomer has been reported in rodent and human tissues (Wolosker *et al.* 1999). Moreover, a sodium-independent neutral amino acid transporter encoded by *asc-1* displays a high affinity for D-serine as well as L-serine (Fukasawa *et al.* 2000), although D-serine is also taken up into brain tissues in a sodium-dependent manner (Yamamoto *et al.* 2001; Javitt *et al.* 2002; Ribeiro *et al.* 2002).

However, little is known about the exact molecules involved in the metabolic or functional processes involving endogenous brain D-serine. One approach to clarifying these molecules is to identify transcripts that respond to D-serine because neuroactive substances commonly influence the expression of the molecules specifically associated with their metabolism and function. To this end, we have successfully applied a differential cloning technique, RNA arbitrarily primed PCR (RAP–PCR) (Welsh *et al.* 1992), and recently cloned a novel D-serine-, but not L-serine-, responsive transcript, *dsr-1*, from rat neocortex (Tsuchida *et al.* 2001). The predicted *dsr-1* products include a proton-ATPase-like amino acid sequence and are suggested to regulate the uptake or release of D-serine (Tsuchida *et al.* 2001). We have therefore extended the exploration strategy to include other possible key genes in the brain D-serine system.

Materials and methods

Animals and reagents

All animal experiments were performed in strict accordance with the guidance of the Tokyo Medical and Dental University Graduate School, and were approved by the Animal Investigation Committee of the Institute. Male Wistar rats (Clea Japan, Inc., Tokyo, Japan) at postnatal day (PD) 8 (13–20 g) and 50 (200–230 g) were used. The animals were housed at 22.0 ± 0.5°C in a humidity-controlled room under a 12-h light–dark cycle and had free access to food and water.

[α-^{32}P]dCTP and [α-^{32}P]UTP were purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA). D- and L-Serine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and dissolved in saline for intraperitoneal (i.p.) injection. For these injection experiments, the rats at PD8 were mainly used because the permeability and accumulation of neutral amino acids in the brain after systemic administration was much less efficient in adult rats compared with that in infant rats, as demonstrated previously (Lefauconnier and Trouve 1983; Takahashi *et al.* 1997; Tsuchida *et al.* 2001). The other chemicals were of ultrapure grade and commercially available.

RNA fingerprinting by RAP–PCR

The rats were received i.p. D- or L-serine (9 mmol/kg bodyweight) dissolved in saline, or saline alone (vehicle) in a volume of 80–120 μL [bodyweight (g) × 6 μl] and killed by cervical dislocation 3 or 15 h later. Because our previous study (Takahashi *et al.* 1997) indicated that systemic administration of a single large dose of D-serine (9 mmol/kg) caused a rapid and linear increase in the neocortical D-serine content until 6 h after injection (steep rise phase) and a constant and prolonged increase in D-serine level from 6 to 15 h after injection (plateau phase), we chose these 3- and 15-h time points as being representative of the two different phases of the increase in D-serine. After i.p. injection, the L-serine content in the neocortex rapidly and dramatically increased, peaked at 3 h, and returned to control level at around 15 h after injection (Takahashi *et al.* 1997).

Following removal of the brain, the cerebral neocortex (dorsal part of the cerebral cortex divided along the rhinal fissure) was rapidly dissected out on ice, immediately frozen in liquid nitrogen and stored at −80°C until use. Total RNA was extracted using the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi 1987) and converted to single-stranded cDNA by reverse transcriptase with random hexamer primers (SuperScript First-Strand Synthesis System for RT–PCR; Invitrogen Corp., Carlsbad, CA, USA). In preparation of the pooled sample for RNA fingerprinting, eight of an equal amount (40 μl; 1 ng/μl) of the resulting cDNA solution obtained from each rat were combined together in each experimental group (animals treated with D-serine, L-serine or saline for 3 or 15 h). The pooled samples were serially diluted in TE buffer containing 10 mM Tris-Hcl (pH 8.0) 1 mM EDTA and used as a template for PCR using a set of rhodamine-labeled 12-mer arbitrary primers (A63: 5′-CAGGTGTGGGTT-3′; for both the 5′- and 3′-primer). Rhodamine reagent (TAMRA-NHS/DMSO; Applied Biosystems, Foster City, CA, USA) was used to label the 5′-amino-modified oligonucleotide. The reaction was terminated by 0.1 M triethylammonium acetate, and the labeled nucleotide was purified by gel filtration.

The PCR protcol comprised 94°C for 2 min, 40°C for 5 min and 72°C for 5 min for the first cycle; 94°C for 30 s, 40°C for 2 min and 72°C for 1 min for 34 cycles; and then 72°C for 5 min for extension. The PCR products were separated in a 5% denaturing polyacrylamide gel including 7 M urea and 1 × TBE (90 mM Tris-borate and 2 mM EDTA). The cDNA bands separated in the gel were visualized and analyzed using a fluorescence image analyzer (FMBIO II Multi-View; Hitachi Software Engineering Co., Ltd, Tokyo, Japan). The cDNA bands that showed a higher intensity in the D-serine-treated group than in any other group were cut out of the gel. The DNA fragments were re-amplified by a second PCR with the same primer set.

The amplified DNA fragments were cloned using a Thymine and Adenine (TA) cloning vector (pGEM-T Easy vector system; Promega, Madison, WI, USA) and sequenced by ABI Prism 3100 (Applied Biosystems). To determine the full-length structure from the isolated DNA fragments, we performed a rapid amplification of cDNA ends (RACE)–PCR with an aliquot of the oligo-dT selected RNA (1 μg) prepared from the neocortex (SMART RACE cDNA amplification kit; Clontech, La Jolla, CA, USA).

Semiquantitative co-amplification RT–PCR

In experiments with the D- or L-serine, or saline injections, the amount of single-stranded cDNA prepared from the neocortical total RNAs of each rat was individually quantified by co-amplification RT–PCR with an endogenous template as internal standard to further control for variations in sampling and processing between samples (Foley et al. 1993; Lombardo and Brown 1996). 28S rRNA was employed as internal control, because neither D- nor L-serine

affects the expression levels of 28S rRNA (Foley et al. 1993; Tsuchida et al. 2001). In brief, RT was performed using 0.2 μg neocortical total RNA from each animal, and the resulting cDNA was suspended in 10 volumes of TE buffer (pH 8.0) for the PCR template. An appropriate portion (71 or 91% for co-amplification with *dsr-2*) of the added primers specific for the 28S rRNA sequence was phosphorylated at their 3′-ends so that the target and the control exponential phases would overlap. The following primers were used: *dsr-2*, 5′-TGAGCCAGGAATTTAGGAAGGTT-3′ (nt 6187-6209) (5′-primer) and 5′-AGCAAATCTGGCCAAGTCTAATG-3′ (nt 6694-6716) (3′-primer), size of PCR product 530 bp (nt 6187-6716); r28S, 5′-CTCGCTGGCCCTTGAAAATCC-3′ [nucleotides (nt) 2570–2590] (5′-primer) and 5′-CCCAGCCCTTAGAGCCAA TCCTTA-3′ (nt 2719–2742) (3′-primer), size of PCR 173 bp (nt 2570–2742: accession V01270). The PCR parameters used were 94°C for 2 min, 61°C for 3 min and 72°C for 5 min for the first cycle; 94°C for 45 s, 61°C for 2 min and 72°C for 3 min for two cycles; 94°C for 30 s, 61°C for 45 s and 72°C for 1 min for 32 cycles; and then 72°C for 5 min for extension. The PCR products were separated by electrophoresis on 3% NuSieve agarose 3 : 1 gel (FMC Bioproducts, Rockland, ME, USA) in 1 × TAE (40 mm Tris-acetate and 1 mm EDTA). The gel was stained with 0.5 μg/mL ethidium bromide for 30 min. The resulting cDNA bands were visualized by UV irradiation and analyzed quantitatively by measuring the optical density with an image analyzer (Lumi-Imager; Roche Diagnostics, Basel, Switzerland). The relative amount of the *dsr-2* transcript was evaluated as the ratio of the optical density of the *dsr-2* band to that of the 28S rRNA band.

Southern and northern blot analyses

For the Southern blot analysis (Toda *et al.* 2000), genomic DNA purified from the rat cerebral neocortex was digested with restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII. The resulting DNA fragments (5 μg) were separated in a 1% agarose gel with 1 × TAE and blotted on to a nylon membrane (Hybond-XL; Amersham Biosciences Corp.) by capillary transfer and fixed by UV cross-linking (Hashimoto *et al.* 1998; Toda *et al.* 2000). The membrane was prehybridized at 42°C for 1 h, and subsequently hybridized with the ^{32}P-labeled cDNA probe for *dsr-2* (Megaprime DNA labeling system; Amersham Corp.) in hybridization buffer (Ultrahyb; Ambion, Inc., Austin, TX, USA) at 42°C for 12 h. The hybridized filter was washed in 0.1 × SSC (20 × SSC: 3 M NaCl and 0.3 M sodium citrate) and 0.1% sodium dodecyl sulfate at 60°C for 60 min. For northern blot analysis, poly(A)$^+$ and poly(A)$^-$ RNA from rat cerebral neocortex were purified using a FastTrack 2.0 kit (Invitrogen Corp.), and separated by electrophoresis in a 1% agarose gel containing 6.3% formaldehyde, then blotted on to a nylon membrane by capillary transfer and fixed by UV cross-linking. After prehybridization at 68°C for 1 h, the blotted filter was hybridized with ^{32}P-labeled antisense RNA probes (Riboprobe systems; Promega) corresponding to 1667 bases (nt 4999–6165) of rat *dsr-2* cDNA and 815 bases (nt 1818–2632) of rat neurexin-3α (*nrxn3α*) cDNA at 68°C for 12 h. The filters were washed in 0.1 × SSC and 0.1% sodium dodecyl sulfate at 68°C for 60 min. In these blotting analyses, the washed filters were exposed to an imaging plate and the signals were visualized using a BAS-2500 image analyzer (Fuji Photo Film Co., Ltd, Tokyo, Japan). The probes for the northern blotting procedures were prepared from the pGEM-T Easy vector.

Analysis of genomic structure surrounding *dsr-2* gene

The hybridization screening of the rat genomic RPCI-31 P1-derived Artificial Chromosome (PAC) library was performed with the *dsr-2* cDNA probe used for the Southern blotting (Woon *et al.* 1998; Osoegawa *et al.* 2000). The isolated PAC genomic DNA clone was purified using a large-size plasmid purification kit (NucleoBond BAC100 kit; Macherey-Nagel GmbH and Co. KG, Düren, Germany), sheared into small DNA pieces, and subcloned into the pUC118 vector as a shotgun library. The genomic structure of *dsr-2* was finally obtained as the contigs constructed by the assembly of the random shotgun sequences.

Determination of tissue distribution by RT–PCR

To examine the tissue distribution of the *dsr-2* and neurexin-3α transcripts, we used the cDNA template prepared from various rat tissues (Rat MTC panel I; Clontech Laboratories, Inc., Palo Alto, CA, USA) for RT–PCR (Toda *et al.* 2000). To determine the subregional distribution in the brain and ontogenic changes, total RNA was extracted from each brain region, and reverse transcribed with random hexamer primers as described above. For *dsr-2*, primers were the same as those used for co-amplification PCR. Those for *nrxn3α* were 5′-ATTTGGATGATGGTGGTGTCTGTG-3′ (nt 959–982) (5′-primer) and 5′-TCCTCTCGAGCTTTACTTCTACCT-3′ (nt 1518–1541) (3′-primer), size of PCR product 583 bp (nt 959–1541: accession NM_053817); β-actin, 5′-CTGGGACGATATGGAGAA-GATTTG-3′ (nt 315–338) (5′-primer) and 5′-GGCATCG-GAACCGCTCATTGCCGA-3′ (nt 830–853) (3′-primer), size of PCR product 539 bp (nt315–853: accession NM_031144); GAPDH, 5′-TGGTGAGTATGTCGTGGAGTCT-3′ (nt 343–364) (5′-primer) and 5′-AATGGGAGTTGCTGTTGAAGTC-3′ (nt 923–944) (3′-primer), size of PCR product 602 bp (nt 343–944: accession BC059110); NMDA receptor NR2B subunit, 5′-ATCGGCCTGCC-CTCCTCCAAACATAGC-3′ (nt 3130–3156) (5′-primer) and 5′-GGGCCCACCTCCACTGACCGAATCTC-3′ (nt 3548–3573) (3′-primer), size of PCR product 444 bp (nt 3130–3573: accession M91562); DAO, 5′-GCTGGGGAACTGGAGCGAGCTAAACAG-3′ (822–848) (5′-primer) and 5′-CTGGGGCTGGGGGAGGGAAT-CATCA-3′ (nt 1204–1229) (3′-primer), size of PCR product 408 bp (nt 822–1229: accession AB003400).

The PCR parameters were as follows: *dsr-2* and neurexin-3α: 94°C for 3 min, 55°C for 2 min and 72°C for 3 min for the first cycle; 94°C for 30 s, 55°C for 1 min and 72°C for 2 min for two cycles; 94°C for 30 s, 55°C for 45 s and 72°C for 90 s for 34 (for *dsr-2*) or 29 (for *nrxn3α*) cycles; and 72°C for 5 min for extension. β-Actin: 94°C for 2 min, 55°C for 3 min and 72°C for 5min for the first cycle; 94°C for 45 s, 55°C for 2 min and 72°C for 3 min for two cycles; 94°C for 30 s, 55°C for 45 s and 72°C for 1 min for 21 cycles; and then 72°C for 5 min for extension. GAPDH: 94°C for 3 min, 55°C for 2 min and 72°C for 3 min for the first cycle; 94°C for 30 s, 55°C for 45 s and 72°C for 3 min for 26 cycles; and then 72°C for 5 min for extension. NR2B: 94°C for 3 min, 60°C for 2 min and 72°C for 3 min for the first cycle; 94°C for 30 s, 60°C for 1 min and 72°C for 90 s for two cycles; 94°C for 30 s, 60°C for 45 s and 72°C for 90 s for 30 cycles; and then 72°C for 5 min for extension. DAO: 94°C for 3 min, 55°C for 2 min and 72°C for 3 min for the first cycle; 94°C for 30 s, 55°C for 1 min and 72°C for 90 s for two cycles; 94°C for 30 s, 55°C for 45 s and 72°C for 90 s for 31 cycles; and then 72°C for 5 min for extension.

Statistical analysis

The results were usually expressed as the mean ± SEM of data obtained from eight determinations. Statistical analyses were performed using one-way ANOVA followed by Scheffé's multiple comparison test.

Results

Isolation of the D-serine transcript *dsr-2*

To identify the transcript that exhibits a response to D-serine, we performed RAP–PCR using neocortical samples prepared from 8-day-old rats, 3 and 15 h after i.p. injection of D- and L-serine (9 mmol/kg) and saline. In previous experiments, we confirmed that these amino acids had efficiently accumulated in the cerebral neocortex at these times after systemic administration (Takahashi *et al.* 1997). Under these conditions, we screened the responsive genes using several arbitrary primers. As shown in Fig. 1(a), using a rhodamine-labelled primer (A63), we detected an amplified DNA band that was enhanced by the injection of D-serine, but not L-serine, at 3 h. The DNA was isolated from the gel, and designated as *dsr-2* (D-serine *r*esponsive transcript-2). Using a semiquantitative co-amplification PCR method with 28S rRNA (Tsuchida *et al.* 2001; Kajii *et al.* 2003), we confirmed that levels of *dsr-2* mRNA in the cerebral neocortex had significantly increased by 3 h after administration of D-serine in a stereoselective manner (Fig. 1b).

Structural analysis of *dsr-2* gene

Based on the structural analysis of cDNA by RACE–PCR and genomic walking, we determined the 7199 bases of the nucleotide sequence of the *dsr-2* cDNA, which consisted of a single exon (Fig. 2). The deduced 111 amino acids of the peptide sequence were predicted by a computer-based open reading frame (ORF) search analysis using the GENETYX program (GENETYX version 7.0; Genetyx Corp., Tokyo, Japan). However, the translational initiation sequence did not satisfy the Kozak consensus sequence. Further investigation is required to confirm the N-terminus of the predicted protein. The above obtained nucleotide sequence has been deposited at the DNA Data Bank of Japan (DDBJ) (Accession No. AB200323).

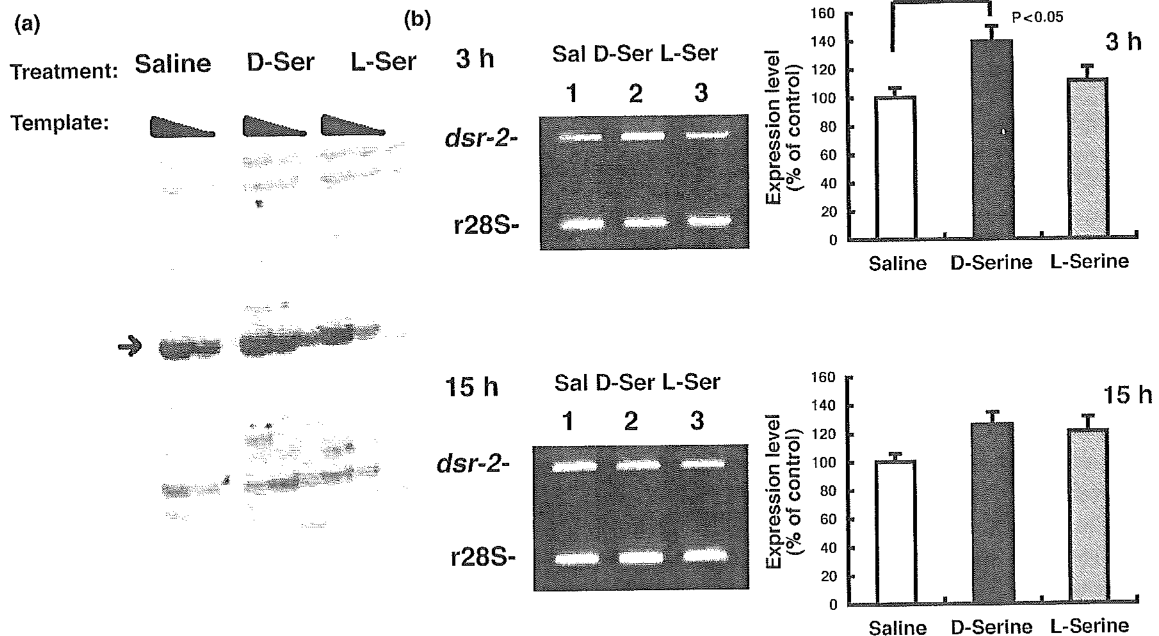

Fig. 1 Isolation of D-serine-regulated transcript *dsr-2* from rat brain. (a) Detection of *dsr-2* in the neocortex of the infant rat 3 h after i.p. injection of D-serine, L-serine or saline by RNA fingerprinting using RAP–PCR. The serially diluted neocortical cDNA templates (concentrations at × 4, × 2 and × 1 from left to right in each group) were amplified with a set of rhodamine-labeled primers of the same sequence (A63: 5′-CAGGTGTGGGTT-3′). The arrow indicates the signal corresponding to the *dsr-2* DNA fragment that was enhanced by D-serine. (b) Effects of systemic administration of D- and L-serine on the expression of *dsr-2* transcript in the neocortex of infant rat. The neocortex was dissected out 3 or 15 h after systemic injection of D- or L-serine, or saline. The level of *dsr-2* was determined quantitatively by the co-amplification RT–PCR method with 28S rRNA as an endogenous control. Representative gel images of the resulting *dsr-2* and r28S bands are shown in the left panel (phosphorylation rate of the r28S primers was 71%). Results are mean ±SEM of eight individual values per experimental group (3-h or 15-h D-serine, L-serine or saline treatment) expressed as a percentage of the respective saline-treated control value [control values: 3 h, 0.87 ± 0.06; 15 h, 1.05 ± 0.06 (phosphorylation rate of the r28S primers was 91%]. $*p < 0.05$ (ANOVA followed by Scheffe's multiple comparison test).

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M Q R Y T K I H S D K F F L D F S P S K S S S I Y V F S S V Y M T I P I A V F

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R L I S Y K N E M Y S K S I L T S I C N I L R T N Y S L S L S L S L S L S L C V C V C V C D M F V L

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C L H M S V S V Y M T I N V G N R A R G R H

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TGAGTCTCACTCAGGGAAGATTCACAAATTACAACATGCTAATCTAGAACCAGAGATAGCACTTTGGATTAGATCTAATCACAATGTCATGGATTTGATCCCTACTGTCATACTGTCATAGAAACAAGATATGGTTGGTGCATACCTAAA 7050
CTCTCAGCACTTGAAAAATGAACGTGAGAAGAAGTTTGAAATCCTCACCTACACGATGAGTTTGAAGGGAGCTGTGAGACATAGGACACCATCCCAAAAATCAGCCCTCCCACATTCCTCAATGAGAAAGAGAGAGAGAGAGAGAGA 7199

Fig. 2 Nucleotide sequence of *dsr-2* cDNA. The 7199-bp sequence of *dsr-2* cDNA is shown with the deduced amino acid sequence, predicted by the computer-based ORF search analysis using the GENETYX program (GENETYX version 7.0; Genetyx Corp., Tokyo, Japan). The nucleotides contained in the ORF are marked with fine underlines. The nucleotide sequences initially primed by the arbitrary primer A63 are underlined in bold. An atypical poly(A) signal before the poly(A) stretch is indicated as an open box. The nucleotide sequence obtained has been deposited at the DDBJ (Accession no. AB200323).

In Southern blotting using 1667 bases of the cDNA probe for *dsr-2*, we detected a single band at 4.4, 12.7 and 2.3 in the *Bam*HI-, *Eco*RI- and *Hin*dIII-digested genomic DNA of the rat respectively (Fig. 3a). With the same probe, a single band at 9.5 kb was detected in *Eco*RI-digested mouse genomic DNA (data not shown). These results indicate that the *dsr-2* gene exists as a single copy in both the rat and mouse genomes. Using the same cDNA probe, we screened a rat genomic library to clarify the genomic structure surrounding the *dsr-2* gene locus. We isolated a positive clone with an insert size of 176 kb from the rat PAC genomic library (Woon *et al.* 1998) and determined the entire primary structure by shotgun sequencing. These analyses, together with the Basic Local Alignment Search Tool (BLAST) database search of rat genomic DNA, revealed that the *dsr-2* gene maps to the opposite strand of the large intron (354 kb) between exon 5 and exon 6 of the *nrxn3α* gene in rat chromosome 6. The rat *nrxn3α* gene consists of 7009 nucleotides of mRNA, extends to the large range of chromosome 6q24-31 (1.63 Mbp) and has multiple alternative splicing sites. The BLAST search further revealed that the mouse or human homologous nucleotide sequence of the rat *dsr-2* gene also mapped to the opposite strand of the similar large intron of the mouse *nrxn3* gene (NM_172544) on chromosome 12 (NT_039552) or that of the human *nrxn3* gene (NM_004796) on chromosome 14 (NT_086807).

Detection of *dsr-2* mRNA in the CNS

Because the polyadenylation signal of the *dsr-2* transcript was found to be atypical, we examined the mRNA signals by the northern blot analysis, not only in the poly(A)$^+$ RNA fraction but also in the poly(A)$^-$ RNA fraction prepared from the neocortex. With a specific antisense RNA probe for the *dsr-2* transcript constructed from the genomic *dsr-2* sequence, we detected a single band at

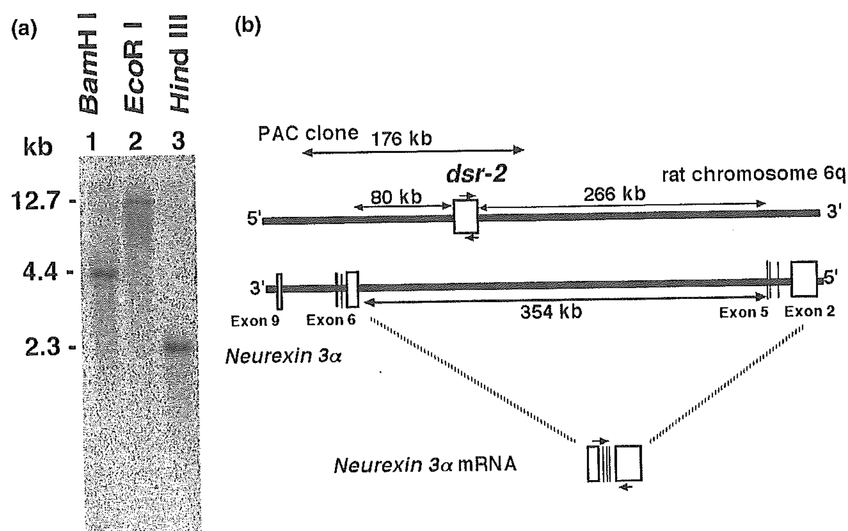

Fig. 3 Genomic organization of *dsr-2*. (a) Southern blot analysis of *dsr-2*. Rat genomic DNA was isolated from adult rat neocortex and digested by one of three restriction enzymes. A single band at 4.4 kb, 12.7 kb and 2.3 kb was detected with the DNA digested by *Bam*HI, *Eco*RI and *Hin*dIII respectively. (b) Genomic structure of *dsr-2* in the rat. The genomic organization of *dsr-2* and *nrxn3α* is illustrated based on the data obtained by DNA sequencing of the corresponding PAC genomic clone and BLAST database analyses. The *dsr-2* gene is located on the reverse strand within a large intron of the *nrxn3α* gene mapped to rat chromosome 6q24-31. The short arrows indicate the positions of primers used for RT–PCR.

7.1 kb in the northern blot analysis of the poly(A)$^+$, but not of the poly(A)$^-$, RNA fraction (Fig. 4a). The size of *dsr-2* transcripts coincided with that of the obtained *dsr-2* cDNA, confirming that the cloned cDNA is full-length. The corresponding sense probe for the *dsr-2* transcript failed to produce any hybridization signals. With the antisense probe for the *nrxn3α* transcript, which is constructed from the opposite strand of the genomic *dsr-2* sequence, a doublet of bands around 9.5 kb was observed (Fig. 4a) as reported previously (Ushkaryov and Südhof 1993). The *nrxn-3* sense probe did not detect any messages from the *dsr-2* strand (Fig. 4a).

Distribution of *dsr-2* transcript in forebrain areas of adult rat

The tissue distribution of *dsr-2* and *nrxn3α* mRNAs was examined in the adult rat (PD50) by RT–PCR (Fig. 4b). Basal expression of the *dsr-2* transcript was exclusively detected in the brain. Basal levels of the *nrxn3α* transcript were detected in the brain and testis. The reliability of the present RT–PCR method was confirmed by the ubiquitous distribution of high levels of β-actin mRNA.

The regional distribution of *dsr-2* mRNA in the brain was examined by RT–PCR assay in the adult rat (Fig. 5a). Expression of the *dsr-2* transcript was predominant in the forebrain (cortex, limbic forebrain, striatum and hippocampus), less in the midbrain (thalamus, hypothalamus and midbrain), and even lower in the hindbrain (pons–medulla and cerebellum). This characteristic distribution closely resembled that of free D-serine in the rat brain as determined

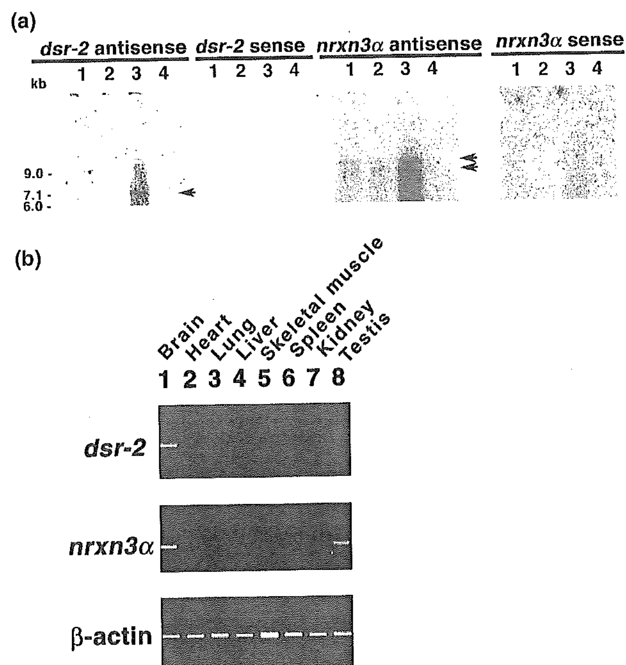

Fig. 4 Expression of *dsr-2* mRNA in brain. (a) Northern blot analysis of *dsr-2* and *nrxn3α*. The mRNA was extracted from adult rat cerebral neocortex and hybridized with *dsr-2* (antisense and sense) and *nrxn3α* (antisense and sense) riboprobes. Lane 1, 10 μg total RNA; lane 2, 2 μg poly(A)$^+$ RNA; lane 3, 20 μg poly(A)$^+$ RNA; lane 4, 10 μg poly(A)$^-$ RNA. The arrows indicate the specific bands detected in the poly(A)$^+$ fraction. (b) Tissue distribution of *dsr-2* and *nrxn3α* transcripts. The basal expression of *dsr-2*, *nrxn3α* and β-actin mRNAs in brain and peripheral tissues was determined by RT–PCR.

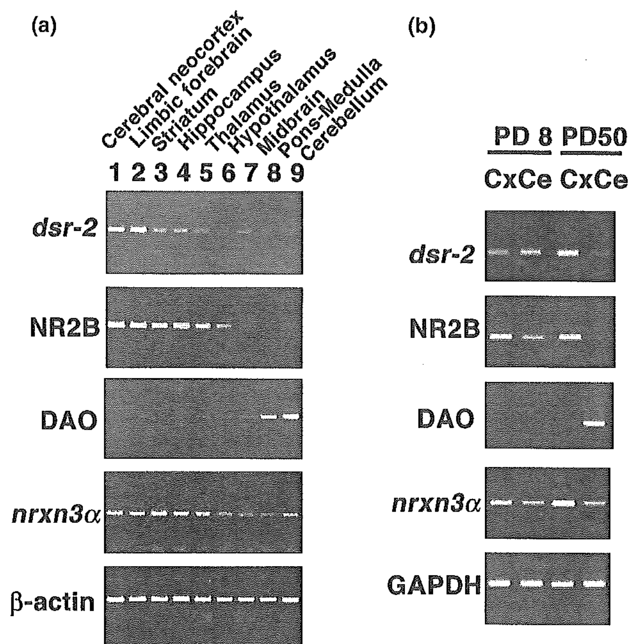

Fig. 5 Ontogenic change in distribution of *dsr-2* transcript in the rat CNS. (a) Subregional distribution of *dsr-2* mRNA in the adult rat brain. The basal expression of *dsr-2*, NR2B, DAO and *nrxn3α* mRNAs was determined semiquantitatively by RT–PCR. (b) Ontogenic changes in the distribution pattern of *dsr-2* mRNA. The basal expression of *dsr-2*, NR2B, DAO and *nrxn3α* mRNAs was determined semiquantitatively by RT–PCR in the infant (PD8) and adult (PD50) rat cerebral neocortex (Cx) and cerebellum (Ce).

by HPLC (Hashimoto *et al.* 1993c) and by immunohistochemistry (Schell *et al.* 1995). Based on previous studies that involved *in situ* hybridization and immunohistochemical analyses (Monyer *et al.* 1992; Nakanishi 1992; Horiike *et al.* 1994), it was speculated that D-serine and the NR2B subunit would display a similar distribution pattern and that DAO would be distributed in a complementary manner to the two molecules. Results of analysis of the distribution of the NR2B and DAO transcripts in various brain areas using RT–PCR supported this presumption (Fig. 5a), suggesting that the *dsr-2* gene product may be involved in synaptic functions regulated by the D-serine/NMDA receptor NR2B-mediated signaling. The basal expression level of the *nrxn3α* transcript was also predominant in the forebrain. However, we detected a relatively large amount of *nrxn3α* mRNA in rat cerebellum. The reliability of the RT–PCR method was verified by the ubiquitous distribution of β-actin mRNA.

Ontogenetic changes in the distribution pattern of *dsr-2* transcript in the rat brain

During development of the rat, endogenous D-serine and the NR2B subunit were both detected transiently (from first to third week after birth) at high levels in the cerebellum (Watanabe *et al.* 1992; Hashimoto *et al.* 1995b; Schell *et al.* 1997). In this study, therefore, the expression of *dsr-2* mRNA was examined in the cerebral neocortex and cerebellum of both infant (PD8) and adult (PD50) rats by RT–PCR. In the infant rat, the cerebellum showed much higher expression of *dsr-2* mRNA than the cerebral neocortex, whereas the adult neocortex and cerebellum showed intense and no detectable expression of the transcript respectively (Fig. 5b). This ontogenic change in the distribution pattern was again similar to that of NR2B mRNA and complementary to that of DAO mRNA. In contrast, there was no change in the distribution pattern of *nrxn3α* mRNA during postnatal development. The fact that no regional and developmental differences were seen in GAPDH mRNA expression attested to the reliability of the RT–PCR method.

Discussion

Using an RNA fingerprinting technique, we isolated a novel and D-serine up-regulated transcript designated as *dsr-2* from the rat neocortex. Systemic administration of D-serine, but not L-serine, enhances the neocortical expression of *dsr-2* mRNA. The *dsr-2* gene is located on the reverse strand within an intron of the *nrxn3α* gene mapped to rat chromosome 6q24-31 and is exclusively transcribed in the brain. The regional distribution of the basal expression of *dsr-2* and its ontogenic changes are closely correlated with those of the free D-serine content and of the NMDA receptor R2B subunit.

Detection of a single band of 7.1 kb by northern blot hybridization with a specific riboprobe for *dsr-2* (Fig. 3a), the RT–PCR results and database search (data not shown) support the view that there are no apparent splicing variants of the *dsr-2* transcript. Sequence analysis of the cloned *dsr-2* cDNA indicates that *dsr-2* mRNA has an ORF that is predicted to encode 111 amino acids. Because the deduced amino acid sequence contains no consensus motifs, the exact physiological significance of the predicted protein remains unclear.

Modification of *dsr-2* expression is likely to be related to NMDA receptor-mediated glutamate transmission because glycine site-mediated facilitation of the NMDA receptor is caused by D-serine. The possible functional link of *dsr-2* with D-serine and NMDA receptors is further supported by the brain-selective and D-serine- and NR2B subunit-like distribution of the basal expression of *dsr-2* in infant and adult rat brains. The pharmacological, neuroanatomical and developmental correlates also indicate that *dsr-2* and its protein product may play a physiological role in control of the metabolism and function of D-serine and NMDA receptors.

Genomic walking, genome library screening and a database search revealed that the entire length of the *dsr-2* gene lies within the opposite DNA strand of the 354-kb intron of the *nrxn3α* gene that encodes highly polymorphic neuron-specific cell-surface proteins involved in the regulation of synaptic neurotransmission (Missler *et al.* 2003). This unique structural

relationship between the two genes, which is conserved in the mouse and human, could reflect their functional association.

The *dsr-2* transcript might act as a natural antisense regulator of *nrxn3α* expression. It has recently been appreciated that changes in mRNA half-life and translation can be mediated not only by RNA-binding proteins but also by sense–antisense RNA interactions (Lehner *et al.* 2002; Carmichael 2003). For instance, translation of the neural nitric oxide synthase protein from its mRNA has been shown to be inhibited by the corresponding antisense region of the pseudogene (Korneev *et al.* 1999; Korneev and O'Shera 2002). The resulting RNA duplex would prevent the sense RNA from interacting with diverse cellular components required for a normal sense expression. Alternatively, the duplex may represent substrates for double-stranded RNA-specific enzymes (Vanhée-Brossollet and Vaquero 1998). Because the DNA sequence of the *dsr-2* gene is complementary to that of a part of the *nrxn3α* intron between its 5 and 6 exons, the *dsr-2* transcript might influence the editing and maturation of premature *nrxn3α* mRNAs by hybridizing to the complementary sequence. However, it is unlikely that the *dsr-2* transcript would solely be a specific antisense regulator of the *nrxn3α* gene, as expression patterns of *dsr-2* and *nrxn3α* mRNAs are not always parallel in the brain and periphery; in adult rats, substantial expression of *nrxn3α*, but not *dsr-2*, mRNA is detected in the cerebellum and testis, whereas the cerebellum exhibits intense expression of these genes during development.

It is also possible that the *dsr-2* RNA might be required for *trans*-splicing variants of *nrxn3α*. *Trans*-splicing is an intermolecular reaction between a splice donor and a splice acceptor in two separate mRNAs (Pirrotta 2002) and has recently been observed in editing mammalian gene transcripts including rat carnitine octanoyltransferase, rat voltage-gated sodium channel and rat Sp1 mRNAs (Caudevilla *et al.* 1998; Akopian *et al.* 1999; Takahara *et al.* 2002). The above possibility is raised by the hypothesis that, in the particular variation upon splicing, the RNAs on opposite strands are independently transcribed but the transcribed regions overlap, therefore resulting in the presence of complementary sequences in the primary transcripts to be *trans*-spliced. A lack of reports of *nrxn3α* variants containing the nucleotide sequence of *dsr-2* and the differential expression of the two genes in the testis and cerebellum, however, argue against the occurrence of *trans*-splicing.

Because the distribution of the mRNA signals of *dsr-2*, NR2B and *nrxn3α* are roughly similar in the brain areas examined, with the exception of the cerebellum, and because α-neurexin (including neurexin-3α)-deficient mice exhibited a decreased NMDA receptor-dependent postsynaptic current (Kattenstroth *et al.* 2004), the sense–antisense transcription in the *dsr-2–nrxn3α* locus is implicated in the regulation of *dsr-2* expression and NMDA receptor-directed D-serine signaling in the brain. Further investigation is needed to clarify these possibilities as well as the physiological role of *dsr-2*; *dsr-2* gene-targeted mice are currently being prepared.

In conclusion, we have demonstrated that rat brain expresses a novel and apparently coding antisense transcript, *dsr-2*, that is induced by D-serine. The D-serine-responsive nature, and brain-selective, D-serine- and NR2B subunit-related distribution, of *dsr-2* mRNA are consistent with the idea that *dsr-2* may participate in the control of D-serine metabolism and function and of NMDA receptor-mediated signaling in the mammalian brain. Genomic analysis indicates that the *dsr-2* mRNA is derived from the opposite strand of an intron of the *nrxn3α* gene, suggesting a regulatory role of sense–antisense transcription in the interactions between *dsr-2*, *nrxn3α*, D-serine and the NMDA receptor.

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Current Topics

D-Amino Acid Biosystem

Metabolism and Functional Roles of Endogenous D-Serine in Mammalian Brains

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It has now been well established that D-serine, a coagonist for the *N*-methyl-D-aspartate (NMDA) glutamate receptors (NR1/NR2 type), is maintained at a high concentration in mammalian brains for life and shows a brain-selective and NMDA receptor R2B subunit-related distribution, overturning the hitherto generally accepted theory that D-amino acid is not always present in mammalian tissues. D-Serine in the brain has been shown to be contained in both the glia and neurons and to have specific processes of biosynthesis, extracellular release, uptake, and degradation. Moreover, the selective elimination of D-serine reduces the NMDA receptor-mediated intracellular signaling and long-term potentiation of synaptic connections. Together with the anti-psychotic and anti-ataxic property of D-serine and the pivotal roles of the NMDA receptor in divergent higher brain functions, these observations support the view that the D-amino acid may be involved as an endogenous modulator for the NMDA receptor in various neuropsychiatric functions and their pathological conditions.

Key words brain; neuropsychiatric disorder; *N*-methyl-D-aspartate (NMDA) glutamate receptor; D-serine

The discovery of endogenous D-serine in the mammalian brain between 1990 and 1991 by the present author with my collaborators at the National Institute of Neuroscience, NCNP, in Tokyo and other institutes, was a consequence of my research project on the pathophysiology and novel pharmacotherapy of schizophrenia using D-serine and D-alanine, which facilitate the *N*-methyl-D-aspartate (NMDA) glutamate receptor function *via* its glycine site (Fig. 1), by noting the induction of schizophrenia-like psychosis by the glutamate receptor antagonists such as phencyclidine (PCP).[1—5] According to this author's idea to overcome the low ability of these polar D-amino acids to cross through the blood brain barrier (BBB) that their apolar compounds could easily permeate the BBB and improve the schizophrenic symptoms by their systemic administration, Dr. Hibino at Nippon Oil and Fats, Co., Ltd., designed and synthesized for our experiments, *N*-myristoyl-D-serine and *N*-myristoyl-D-alanine, which were shown to ameliorate an animal model of schizophrenia, PCP-induced abnormal behavior, following their intraperitoneal injection.[3,6] During the process of the verification of the expected presence of free D-serine or D-alanine in the brain of animals treated with their fatty acid compounds by collaboration with the late Dr. Hayashi at the National Institute of Neuroscience and Dr. Fujii at Tsukuba University, we provided the first evidence that free D-serine is constantly maintained at a high concentration in the mammalian brain[7,8] although D-amino acids were believed to be uncommon in mammalian tissues based on previous studies.[9] Through immediate confirmations[10,11] of our findings, subsequent studies on endogenous D-serine have been gradually extended in a variety of aspects. In this article, the present status and future problems are discussed.

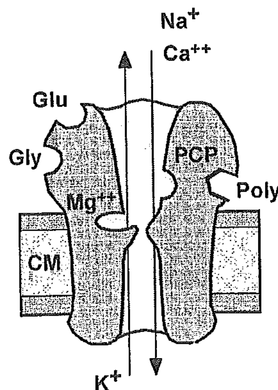

Fig. 1. Schematic Representation of the NMDA Receptor Ion Channel (NR1/NR2 Type)

The NMDA receptor complex has the multiple regulatory binding sites for glutamate (Glu), glycine and D-serine (Gly), magnesium ions (Mg^{++}), phencyclidine (PCP), and polyamine (Poly). This heteromeric receptor consists of an NR1 subunit (there are various variants) and at least one of 4 types of the NR2 subunits, A-D. Gly is considered to be present on NR1 and Glu on NR2. The more recently identified NR3 subunit could have regulatory sites that differ from those shown in this scheme.

1. DISTRIBUTION AND METABOLISM OF ENDOGENOUS D-SERINE IN THE MAMMALIAN BRAIN

1) Distribution In mature rats, D-serine is predominantly concentrated in the brain,[8] and its contents in the spinal cord, each peripheral tissue, or the blood are very low[8] (but high in the urine[12]). The D-serine distribution in the brain is also uneven, showing high concentrations in the forebrain areas including the cerebral cortex, hippocampus, striatum, and limbic forebrain, moderate to low concentrations in the diencephalon and midbrain, and trace levels in the pons-medulla and cerebellum.[8] The forebrain-preferred distribution of D-serine is positively correlated with those of the glutamate, PCP, and glycine sites of the NMDA receptor and particularly with that of the NMDA receptor R2B subunit mRNA.[8,13] These characteristics of the endogenous D-serine distribution have been ascertained by subsequent biochemical[11,14,15] and immunohistochemical studies using the anti-D-serine antibody,[16—18] and proved to be common to humans

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