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BRIEF REPORT

A possible association between the −116C/G single nucleotide polymorphism of the *XBP1* gene and lithium prophylaxis in bipolar disorder

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Abstract

Bipolar disorder (BPD) is a severe, chronic, and life-threatening illness, and its pathogenesis remains unclear. Recently, a functional polymorphism (−116C/G) of the X-box binding protein 1 (*XBP1*) gene was reported to be a genetic risk factor for BPD. Moreover, the endoplasmic reticulum stress responses were impaired in cultured lymphocytes from BPD patients with the −116G allele and only valproate rescued such impairment among three major mood stabilizers. In this context, we hypothesized that BPD patients with different genotypes respond differently to mood stabilizers. We investigated the association between the −116C/G polymorphism of the *XBP1* gene and lithium response in Japanese patients with BPD. We found that lithium treatment is more effective among BPD patients with the −116C allele carrier than in patients homozygous for the −116G allele. The association between the −116C/G polymorphism and clinical efficacy of mood stabilizers should be further investigated in a prospective study with a larger sample.

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Key words: Bipolar disorder, lithium, SNP (single nucleotide polymorphism), *XBP1*.

Introduction

Bipolar disorder (BPD) is a severe, chronic, and life-threatening illness characterized by recurrent episodes of mania and depression. Despite extensive research, its pathogenesis is still unclear. Lithium is listed as a first-line agent for the treatment of BPD by American Psychiatric Association guidelines (APA, 2002). However, a significant percentage of patients with BPD show partial or no response to lithium treatment (Abou-Saleh, 1987). Psychopathological and biological markers that predict lithium response in BPD are not yet elucidated. Therefore, many researchers explored psychopathological and biological markers for lithium response in BPD, and several genetic markers are considered to be good candidates for lithium response (for reviews, see Gelenberg and Pies, 2003; Ikeda and Kato, 2003).

Recently, a functional polymorphism (−116C/G) of the X-box binding protein 1 (*XBP1*) gene that plays a pivotal role in endoplasmic reticulum (ER) stress response was shown to confer susceptibility to BPD (Kakiuchi et al., 2003). The single nucleotide polymorphism (SNP) in the promoter region of the *XBP1* gene was significantly more common in Japanese patients with BPD [odds ratio (OR) 4.6] and over-transmitted to affected offspring in trio samples of the NIMH Bipolar Disorder Genetic Initiative. The *XBP1*-dependent transcription activity of the −116G allele was lower than that of the −116C allele, and induction of *XBP1* expression after ER stress was markedly reduced in the cell with the G allele. Moreover, valproate rescued the impaired response of the cell with the G allele by inducing *ATF6*, the gene upstream of *XBP1*, although lithium and carbamazepine did not. Based on the observations, we hypothesized that BPD

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patients with different genotypes respond differently to treatment with mood stabilizers such as lithium and valproate.

The aim of the study was to examine the possible association between lithium response and the XBP1 −116C/G polymorphism in patients with BPD.

Methods

Subjects

A total of 66 patients with BPD (20 BP I disorders and 46 BP II disorders) were recruited at Hokkaido University Hospital. They were composed of 38 males and 28 females with a mean age of 50.6 yr (S.D. = 11.9 yr) and a mean age at onset of 34.4 yr (S.D. = 11.4 yr). All subjects were biologically unrelated Japanese. Consensus diagnosis was made for each patient by at least two psychiatrists according to DSM-IV criteria (APA, 1994). The presence of concomitant diagnoses of mental retardation, drug dependence, or other Axis I disorder, together with somatic or neurological illnesses that impaired psychiatric evaluation, represented exclusion criteria. Patients had been treated with lithium carbonate and its serum concentration was maintained between 0.4–1.2 mequiv/l at least for 1 yr. Treatment response to lithium was determined for each patient from all available information including clinical interview and medical records, by at least two psychiatrists according to criteria described by Kato et al. (2000). Briefly, lithium responders were defined as those patients who had less frequent and/or severe relapse, including no relapse, during lithium treatment than prior to lithium treatment. Among 66 patients, 43 patients were determined as responders and 23 patients as non-responders. In the 23 non-responders, 15 patients had been treated with valproate at least for 1 yr. We secondarily evaluated the treatment response to valproate using the same criteria as for response to lithium. After complete description of the study, written informed consent was obtained from every subject. The study protocol was approved by the ethics committees of Hokkaido University Graduate School of Medicine and the National Center of Neurology and Psychiatry.

Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. Genotypes for the −116C/G SNP were determined using the TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2004). Briefly, probes and primers for detection of the polymorphism were: forward primer 5′-CTGTCACTCCGGATGGAAATA-AGTC-3′, reverse primer 5′-ATCCCTGGCCAAAGG-TACTTG-3′, probe 1 5′-VIC-CTCCCGCACGTAAC-MGB-3′, and probe 2 5′-FAM-TCCCGCAGGTAAC-MGB-3′. PCR cycling conditions were: 95 °C for 10 min, 45 cycles of 92 °C for 15 s and 60 °C for 1 min.

Statistical analysis

Difference in clinical features between responders and non-responders to lithium treatment was analysed using the χ^2 tests for categorical variables and the t tests for continuous variables. The presence of Hardy–Weinberg equilibrium was examined by using the χ^2 test for goodness of fit. Genotype and allele distributions between responders and non-responders to lithium treatment were analysed by the χ^2 test for independence. Association between genotype and serum lithium levels was analysed by analysis of variance (ANOVA). All p values reported are two-tailed. Statistical significance was defined at $p<0.05$.

Results

The clinical characteristics of patients with BPD are shown in Table 1. Significant differences were not found in clinical features between patients who were defined as responders and non-responders to lithium treatment. Allele frequencies and genotype distributions of the −116C/G polymorphism of the *XBP1* gene among responders and non-responders to lithium treatment are shown in Table 2. The genotype distributions for the total patients, responders, and non-responders were both in Hardy–Weinberg equilibrium (total patients: $\chi^2=1.19$, d.f. = 1, $p=0.28$; responders: $\chi^2=1.8$, d.f. = 1, $p=0.18$; non-responders: $\chi^2=0.13$, d.f. = 1, $p=0.72$). Serum lithium levels in responders did not differ among *XBP1* genotypes [C/C 0.64 (S.D. = 0.10) mequiv/l; C/G 0.66 (S.D. = 0.24) mequiv/l; G/G 0.53 (S.D. = 0.18) mequiv/l; $F=1.83$, $p=0.17$, ANOVA]. On the other hand, there was a trend towards increased serum lithium levels in non-responders homozygous for the −116G allele [C/C 0.48 mequiv/l ($n=1$); C/G 0.53 (S.D. = 0.16) mequiv/l; G/G 0.69 (S.D. = 0.19) mequiv/l], but it did not reach statistical significance ($t=2.0$, d.f. = 20, $p=0.059$, t test comparing patients with C/G and G/G).

There was a trend towards an increased frequency of the −116C allele in the responders rather than non-responders ($\chi^2=3.72$, d.f. = 1, $p=0.054$; OR 2.18, 95% CI 0.98–4.87). Subsequent Mantel–Haenszel tests showed a differential genotype distributions between

Table 1. Background and clinical characteristics of bipolar (BP) patients

	Lithium-treated patient			
	Total (66)	Responder (43)	Non-responder (23)	Responders vs. non-responders
Sex				
Males	38 (57.6%)	28 (65.1%)	10 (43.5%)	$\chi^2=2.87$, d.f.=1,
Females	28 (42.4%)	15 (34.9%)	13 (56.5%)	$p=0.09$
Diagnosis				
BP I	20 (30.3%)	14 (32.6%)	6 (26.1%)	$\chi^2=0.30$, d.f.=1,
BP II	46 (69.7%)	29 (67.4%)	17 (73.9%)	$p=0.59$
Psychotic features				
Present	7 (10.6%)	6 (14.6%)	1 (4.3%)	$\chi^2=1.46$, d.f.=1,
Absent	59 (89.4%)	37 (85.4%)	22 (95.7%)	$p=0.23$
History of rapid cycling				
Present	10 (15.2%)	4 (9.3%)	6 (26.1%)	$\chi^2=3.28$, d.f.=1,
Absent	56 (84.8%)	39 (90.7%)	17 (73.9%)	$p=0.07$
Medication				
Lithium monotherapy	14 (21.2%)	11 (25.6%)	3 (13.0%)	$\chi^2=1.41$, d.f.=1,
Presence of co-administration[a]	52 (78.8%)	32 (74.4%)	20 (87.0%)	$p=0.24$
				t test
Age (yr)[b]	50.6±11.9	51.1±11.3	49.7±13.1	$t=0.44$, d.f.=64, $p=0.66$
Age at onset (yr)[b]	34.4±11.4	34.0±11.7	35.1±11.0	$t=0.38$, d.f.=64, $p=0.70$
Serum lithium concentration[b] (mequiv/l)	0.62±0.20	0.62±0.21	0.62±0.19	$t=0.03$ d.f.=64, $p=0.98$

[a] Aditional administration of valproate, carbamazepine, antidepressants, antipsychotics are included.
[b] Continuous variables are shown as mean±S.D.

responders and non-responders ($\chi^2=4.30$, d.f.=1, $p=0.038$). Thus, we examined the C allele carriers and non-carriers separately, and found that the C allele carriers were significantly more common in the responder group than the non-carriers ($\chi^2=4.34$, d.f.=1, $p=0.037$; OR 3.00, 95% CI 1.05–8.58).

The genotype distributions among responders and non-responders to valproate treatment are shown in Table 3. There was no association between the −116C/G polymorphism of the *XBP1* gene and response to valproate ($\chi^2=1.25$, d.f.=2, $p=0.54$).

Discussion

We investigated the possible association between the *XBP1* gene and the response to lithium treatment in BPD for the first time. Our results suggest that lithium treatment is more effective in BPD patients with the −116C allele of the *XBP1* gene than in patients homozygous for the G allele.

Kakiuchi et al. (2003) proposed that impaired response against ER stress in BPD patients with the G allele might be one of the possible cellular and molecular pathophysiology of BPD. Among three representative mood stabilizers, only valproate rescued this impairment of ER stress response in cultured lymphocytes, although lithium or carbamazepine did not. These findings suggested that the effectiveness of lithium on BPD patients with the G allele might be weaker than those with the C allele. Our clinical observations were consistent with the proposed mechanisms. A possible explanation for the mechanisms of the better efficacy of lithium treatment in −116C carriers is that −116C carrier patients might have other cellular and molecular impairments, which lithium could influence in the nervous system, e.g. inhibition of glycogen synthase kinase-3, inositol monophosphatase and *N*-methyl-D-aspartate receptor activity, activation of the BDNF/Trk pathway, or enhancement of neurogenesis and neuronal progenitor

Table 2. Genotype and allele frequencies of the C −116G polymorphism of the X box-binding protein 1 (*XBP1*) gene and response for lithium treatment

Response for lithium treatment	Allele frequency		χ^2		Genotype distribution			MH		χ^2	
	C	G	*p* value	OR (95% CI)	C/C	C/G	G/G	*p* value	C/C, C/G	G/G	*p* value	OR (95% CI)
Responders (43)	35 (40.7%)	51 (59.3%)	0.054	2.18 (0.96–3.03)	5 (11.6%)	25 (58.1%)	13 (30.2%)	0.038	30 (69.8%)	13 (30.2%)	0.037	3.00 (1.05–8.58)
Non-responders (23)	11 (23.9%)	35 (76.1%)			1 (4.3%)	9 (39.1%)	13 (56.5%)		10 (43.5%)	13 (56.5%)		
Total patients (66)	46 (34.8%)	86 (65.2%)			6 (9.1%)	34 (51.5%)	26 (39.4%)		40 (60.6%)	26 (39.4%)		

OR, Odds ratio; CI, confidence interval; MH, Mantel–Haenszel.

Table 3. Genotype of the −116C/G polymorphism of the *XBP1* gene and response for valproate treatment in lithium non-responders

Response for valproate treatment	Genotype distribution			χ^2
	C/C	C/G	G/G	*p* value
Responders (7)	1 (14.3%)	2 (28.6%)	4 (57.1%)	0.53
Non-responders (8)	0 (0%)	3 (37.5%)	5 (62.5%)	
Total patients (15)	1 (6.7%)	5 (33.3%)	9 (60.0%)	

proliferation (Chen et al., 2000; Hallcher and Sherman, 1980; Hashimoto et al., 2002a,b; 2003; Klein and Melton, 1996). Recently, it has been reported that chronic lithium treatment increased 78-kDa glucose-regulated protein (GRP78), a molecular chaperone of the heat shock protein 70 family, and showed cyto-protective effects in rat PC12 cells (Hiroi et al., 2005). In this regard, one of therapeutic actions of lithium might be associated with reducing ER stress, including signal transduction by *XBP1*. Although there was no direct evidence suggesting that *XBP1* is involved in the pathway of action of lithium, the −116C allele of the *XBP1* gene may contribute to reduce ER stress more effectively by lithium treatment.

Considering the action of valproate in cells with the −116G allele, it is possible that BPD patients with the −116G allele respond to valproate treatment better than those with the −116C allele. Therefore, we investigated the association between valproate response and the −116C/G polymorphism in non-responders to lithium treatment using the same criteria as for lithium response. However, we did not find any association in our small sample. It has been reported that lithium is effective for classical mania, while valproate is effective for both classical and irritable mania (Swann et al., 2002). In this context, valproate is likely to have a wider treatment spectrum than lithium, which may explain our finding. To clarify the association between the −116C/G polymorphism and treatment response to valproate, an independent and larger sample should be investigated.

After Kakiuchi et al. (2003) showed that the −116G allele was a risk factor of BPD in a Japanese sample, there have been two negative studies investigating American and European samples (Cichon et al., 2004), and a Chinese sample (Hou et al., 2004). Among our sample, the allele frequency of the −116G allele in

patients (0.65) was closer to that in controls (0.64) than that in BPD patients (0.71) in Kakiuchi et al.'s report (2003), although both subjects were of the same ethnicity (Japanese). To conclude whether the −116C/G contributes to the genetic risk factor for BPD in the Japanese population, larger number of BPD patients of Japanese origin should be examined.

On the other hand, two positive association studies between the −116G allele and schizophrenia have been reported (Chen et al., 2004; Kakiuchi et al., 2004). It has been reported that schizophrenia and BPD share several susceptibility loci such as 22q12 where the *XBP1* gene is located (Badner and Gershon, 2002). Therefore, these studies concerning schizophrenia might help to identify a shared pathogenesis of these two mental disorders.

To our knowledge, this is the first report indicating that long-term lithium treatment was more effective in BPD patients with the −116C allele on the promoter region of the *XBP1* gene than in those without the −116C allele. The mechanism of lithium response in the C allele-carrier patients is still unknown, however, it may be related to other mechanisms than dysregulation of ER stress response caused by the −116G allele. The limitations of the current study are retrospective design and small sample size. The association between the −116C/G polymorphism and clinical efficacy of mood stabilizers should be further investigated in a prospective study with a larger sample.

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Statement of Interest

None.

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Inhibition of D-serine accumulation in the *Xenopus* oocyte by expression of the rat ortholog of human 3′-phosphoadenosine 5′-phosphosulfate transporter gene isolated from the neocortex as D-serine modulator-1

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Abstract

D-Serine in mammalian brains has been suggested to be an endogenous co-agonist of the NMDA-type glutamate receptor. We have explored the molecules regulating D-serine uptake and release from the rat neocortex cDNA library using a *Xenopus* oocyte expression system, and isolated a cDNA clone designated as *dsm-1* (D-serine modulator-1) encoding a protein that reduces the accumulation of D-serine to the oocyte. *dsm-1* is the rat orthologue of the human 3′-phosphoadenosine 5′-phosphosulfate transporter 1 (*PAPST1*) gene. The hydropathy analysis of the deduced amino acid sequence of the Dsm-1 protein predicts the 10 transmembrane domains with a long hydrophobic stretch in the C-terminal like some amino acid transporters. The *dsm-1* mRNA is predominantly expressed in the forebrain areas that are enriched with D-serine and NMDA receptors, and in the liver. The transient expression of *dsm-1* in COS-7 cells demonstrates a partially Golgi apparatus-related punctuate distribution throughout the cytoplasm with a concentration near the nucleus. *dsm-1*-expressing oocytes diminishes the sodium-dependent and -independent accumulation of D-serine and the basal levels of the intrinsic D-serine and increases the rate of release of the pre-loaded D-serine. These findings indicate that *dsm-1* may, at least in part, be involved in the D-serine translocation across the vesicular or plasma membranes in the brain, and thereby control the extra- and intracellular contents of D-serine.

Keywords: brain, D-serine modulator-1 gene, D-serine, transporter, NMDA receptor, 3′-phsophoadenosine 5′-phosphosulfate, *Xenopus* oocyte.

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D-Serine, a co-agonist for the NMDA receptor (Danysz and Parsons 1998), has now been well documented to be present at high contents throughout life in mammalian brains by using a variety of detection methods for chiral amino acids (Hashimoto *et al.* 1992a,b; Nagata *et al.* 1992; Hashimoto *et al.* 1993a,b,c; Nagata *et al.* 1994; Chouinard *et al.* 1993; Kumashiro *et al.* 1995), although amino acids had long been assumed to exclusively exist as the homochiral L-form in mammalian tissues in proteins and free amino acid pools (Fujii 2002). Endogenous D-serine is predominantly concentrated in the brain (Hashimoto *et al.* 1993c) with an NMDA receptor R2B subunit-like distribution (Hashimoto *et al.* 1993c, 1995b; Kumashiro 1995; Schell *et al.* 1995, 1997), whereas the peripheral tissues contain far lower levels of the D-amino acid (Hashimoto *et al.* 1993c, 1995b).

These neuroanatomical and functional relationships between D-serine and the NMDA receptor imply that endogenous D-serine may regulate the NMDA receptor-mediated glutamate neurotransmission. This hypothesis is supported by our *in vivo* microdialysis study demonstrating the presence of extracellular D-serine in the NMDA receptor-rich brain

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Abbreviations used: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolylphosphate; DIG, digoxigenin; DMEM, Dulbecco's modified minimum essential medium; *dsm-1*, D-serine modulator-1; FBS, fetal bovine serum; FR, Frog Ringer; MBM, modified Barth's medium; NBT, nitro blue tetrazolium; NGF, nerve growth factor; PAPS, 3′-phsophoadenosine 5′-phosphosulfate; PBS, phosphate-buffed saline; SDS, sodium dodecyl sulfate.

regions (Hashimoto *et al.* 1995a). Furthermore, selective depletion of the endogenous D-serine by D-amino acid oxidase without changes in the levels of glycine, another NMDA co-agonist, has indeed been shown to attenuate the NMDA receptor functions in the rat hippocampal slice preparations (Mothet *et al.* 2000).

Consequently, together with the involvement of the NMDA receptor in divergent higher brain functions and an anti-schizophrenic property of D-serine (Tanii *et al.* 1994; Javitt 2004), the extracellular D-serine levels should be under fine 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.* 1993c; Schell *et al.* 1995), uptake (Wako *et al.* 1995; Hayashi *et al.* 1997; Yamamoto *et al.* 2001; Javitt *et al.* 2002; Ribeiro *et al.* 2002), and degradation by D-amino acid oxidase (Hashimoto *et al.* 1993c; Urai *et al.* 2002) for D-serine in the brain. Wolosker *et al.* (1999) reported the isolation of the serine racemase catalyzing conversion of L- to D-serine with serine dehydratase activity from rodent and human tissues. Interestingly, a sodium-independent neutral amino acid transporter encoded by Asc-1 exhibits a high affinity for D-serine as well as L-serine (Fukasawa *et al.* 2000), while D-serine has also been found to be taken up into the brain tissues in a sodium-dependent manner (Yamamoto *et al.* 2001; Javitt *et al.* 2002; Ribeiro *et al.* 2002).

However, the exact molecules comprising the uptake and release machinery for the regulation of the extracellular D-serine and their relation to the D-serine metabolism in the intracellular structures are largely unknown. Therefore, to obtain insight into the D-serine carrier proteins or their regulators, we have explored molecules that modify tritiated or non-radiolabelled D-serine accumulation into the *Xenopus* oocyte.

Materials and methods

Materials

Type I collagenase from *Clostridium histolyticum* was purchased from Sigma-Aldrich (St Louis, MO, USA). [^{3}H]D-Serine (20–25 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA) and Amersham Biosciences (Piscataway, NJ, USA), respectively. [^{3}H]D-Serine was purified using columns packed with Dowex 1-X 8 (100–200 mesh) resin as previously described (Matoba *et al.* 1997). All other chemicals were ultra-pure grade and commercially available.

Animals

The animal experiments were performed in strict accordance with the guidance of the Tokyo Medical and Dental University, and approved by the Committee for Animal Experiment Ethics of the University.

Construction and screening of rat cerebral neocortex cDNA library

From the male Wistar rat (PD 56) cerebral neocortex, poly(A)$^+$ RNA was purified and size-fractionated by 5–20% sucrose density gradient centrifuge at the SW41Ti rotor (Beckman, Palo Alto, CA, USA) at 25 000 r.p.m. (107 000 *g*) for 12 h. Directional cDNA libraries were constructed from these fractions using pSPORT1 SuperScript Plasmid System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Each library was subdivided into 50 pools, each containing 10 000 clones. The respective cDNA pool was transcribed *in vitro* by T7 RNA polymerase (mMESSAGE mMACHINE, Ambion, Austin, TX, USA), and used for microinjection into *Xenopus* oocytes.

Expression screening in *Xenopus* oocytes

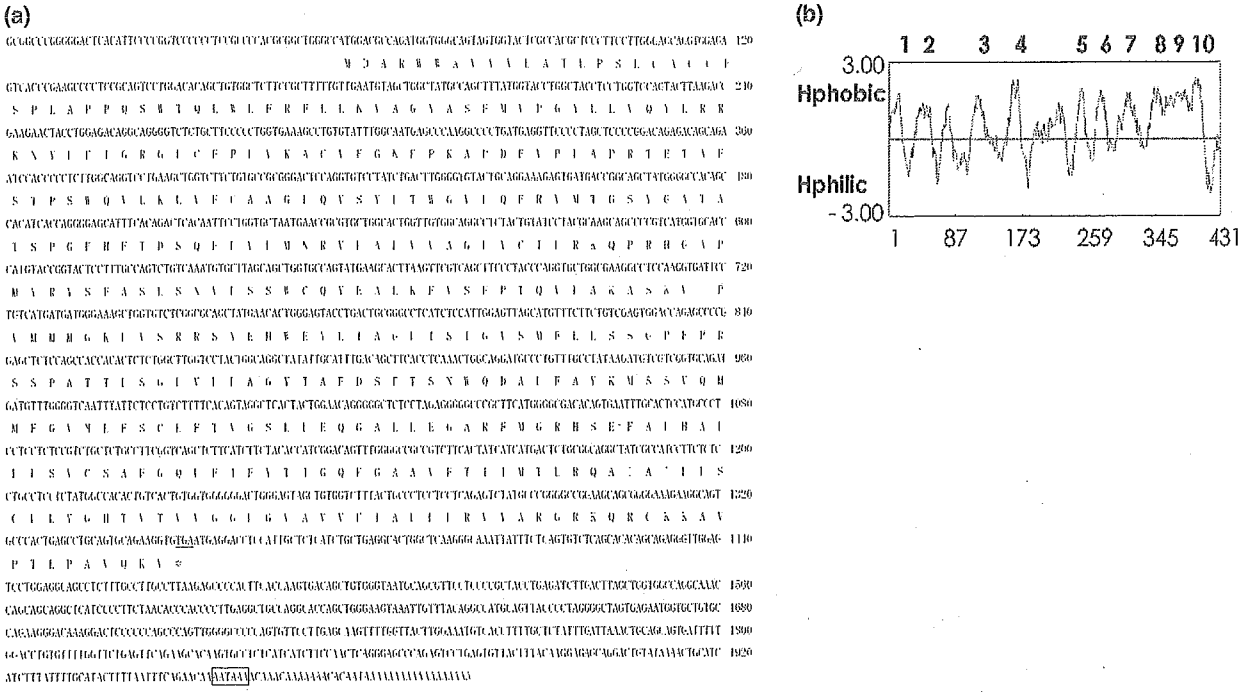
The expression screening assay was performed using collagenase-treated and manually defolliculated oocytes. The cells were microinjected with 50 nL (1 ng/nL dissolved in Rnase-free H_2O) of poly(A)$^+$ RNA, or *in vitro* transcribed cRNA as mentioned above. The same volume of distilled H_2O was used for microinjection as a control. The oocytes were incubated at 20°C in modified Barth's medium (MBM: 80 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 15 mM HEPES, pH 7.4) for 2 days before the assay.

Uptake assay of D-serine

The uptake of D-serine was traced with [^{3}H]D-serine. Groups of 12–20 oocytes were incubated at 26°C for 60 min in 1.5 mL of Frog Ringer (FR: 120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4) containing 10 mM of [^{3}H]D-serine (Utsunomiya-Tate *et al.* 1996) based on the preliminary data that the uptake of [^{3}H]D-serine was found to be linear for at least 4 h and to approach a plateau 8 h after the start of the reaction. At the end of the incubation, non-specific binding of the radiolabelled compound on the cell surface was removed by six times of brief wash with each 10 mL of ice-cold FR solution. Individual oocytes were transferred to a microtube and completely lysed in 100 µL of 0.2% sodium dodecyl sulfate (SDS) in FR by intensive vortex mixing. One millilitre of scintillation fluid (Packard Ultima Gold XR, Parkin-Elmer, Boston, MA, USA) was added to each lysate solution, and the radioactivity was determined by liquid-scintillation counting (LS6500, Beckman). Some fractions induced a 1.5–2-fold increase, while some other fractions a 20–40% decrease in the uptake activity of D-serine into the oocytes. The positive fractions of cDNA library were further sequentially subdivided into the next 50 pools, each of 500–1000 clones. After repeating the same step, one pool containing 100 clones exhibited decreased D-serine uptake reproducibly. Then colonies from the positive pools were seeded individually into the well of 96-well plate to produce a grid system. From the combined testing of rows and columns for D-serine uptake activity, a single positive colony was isolated.

Southern and northern blot analyses

Southern and northern hybridization analyses were performed as previously described (Tsuchida *et al.* 2001.) For the Southern hybridization, 1323 bases of ^{32}P-labelled RNA probe for *dsm-1* (probe 1, corresponding to nucleotide 674–1997 in Fig. 1a) was utilized. The hybridized filter was washed in 0.1 × saline sodium



(c)

```
Dsm-1 (rat)          1:MDA----RWWAVV---------VLATLP---S-----LGACG--E--SPEAPPQSWTQL- 34
PAPST1 (human)       1:MDA----RWWAVV---------VLAAFP---S-----LGAGG--E--TPEAPPESWTQL- 34
SLL (Drosophila)     1:MYAYNKMG-RVPELV1CSF1VVTLLV1HFFSDLLRASLGGYYNQDVTLSQLVESQNSDYA 59
                       *.*  .....       .*.  .  .    **..  .  ..... .....

Dsm 1 (rat)         35:WLFRFLLNVAGYAS FMVPGVLLVQVLRRKAYLETGRGLCFPLV KACVFGMEPKAPDEV 92
PAPST1 (human)      35:WFFRFVVNAAGYAS-FMVPGVLLVQVFRRKNYLETGRGLCFPLV-KACVFGMEPKASDEV 92
SLL (Drosophila)    60:WFLKLLVNCFGYSCVF-VPGFLIVKVYGRIAYLERGNKTFLHKAINMC1TGNSGYDQLDA 118
                       *.....*.**.. *.***.*..* .*.****.*........ ..*.**.... ...

Dsm-1 (rat)         93:PLA-PRTETAESTP-S-W----QV-LKLVFCAAGLQVSYLTWGVLQERVMTGSYGATATSP 146
PAPST1 (human)      93:PLA-PRTEAAETTP-M-W---QA-LKLLFCATGLQVSYLTWGVLQERVMTRSYGATATSP 146
SLL (Drosophila)   119:GTSTADKDRPAASTAPKRTSSQEAVQLLWCFGGLMISYLTWGVLQEKIMTQNYLNFTGES 178
                       ... .... .. ..   .  * ..*..*. **. .**********. .** .*.......

Dsm-1 (rat) .       146:GEHFTDSQFLVLMNRVLALVVAGLVC1LRKQPR---HGAPMVRYSFASLSNVLSSWCQYE 202
PAPST1 (human)      146:GERFTDSQFLVLMNRVLALIVAGLSCVLCKQPR---HGAPMVRYSFASLSNVLSSWCQYE 202
SLL (Drosophila)    179:AK-FKDSQFLVFSNRLLAFLVA-LAYLQWQ-PSPVRHRAPLYKYSYASFSNIMSAWFQYE 235
                       .. *.******. .**.**.  **.* . . ..*.    *.**.*.**.**.**. .*.*.***

Dsm-1 (rat)         203:ALKFVSFPTQVLAKASKV1PVMMMGKLVSRRSYEHWEYLTAGL1S1GVSMFLLSSGPEP- 261
PAPST1 (human)      203:ALKFVSFPTQVLAKASKV1PVMLMGKLVSRRSYEHWEYLTATL1S1GVSMFLLSSGPEP- 261
SLL (Drosophila)    236:ALKFVNFPTQVLAKSCK1IPVMLMGKIMSKAKYESYEYVTALL1SLGMIFFMSGS-SDSS 294
                       *****.*********. .*.****.***. .*.. .**. .**.** ***.*...*...*....

Dsm-1 (rat)         262:RSSPAT-TLSGLVLLAGY1AFDSFTSNWQDALFA-YKMSSVQMMFGVMLFSCLFTVGSLL 319
PAPST1 (human)      262:RSSPAT-TLSGLILLAGY1AFDSFTSNWQDALFA-YKMSSVQMMFGVMFFSCLFTVGSLL 319
SLL (Drosophila)    295:KA-SGVTTLTG1FLLSMYMVFDSFTAWWQGSLFKSYGMTPLQMMCGVMLFSS1FTGASLS 353
                       . ..... **.*.  **..*. .*****.***. .**.  *.*.. .***.***.**. .**. .**.

Dsm-1 (rat)         320:EQGALLRGARFMGRHSEFALHALLLSVCSAFGQLF1FYT1GQFGAAVFT1IMTLRQAIA1 379
PAPST1 (human)      320:EQGALLRGTRFMGRHSGFAAHALLLSHCSACGQLF1FYT1GQFGAAVFT1IMTLRQAFA1 379
SLL (Drosophila)    354:MQGGFMDSLAFATEHPKFVFDMVVLSVCSAVGQLF1YHT1DVFGPVVFT1IMTLRQAVA1 413
                       .**..... .*...*. *.  ....**.*** *****. .**. .**. .********** **

Dsm-1 (rat)         380:LLSCLLYGHTVTVVGGLGVAVVFTALLLRVYARGRK-QRGKKAVPTEPAVQKV       431
PAPST1 (human)      380:LLSCLLYGHTVTVVGGLGVAVVFAALLLRVYARGRLKQRGKKAVPVESPVQKV       432
SLL (Drosophila)    414:MLSCF1YQHS1SLLG1FGVL1VFVA1FLRVYCTQRLRA1RKRAEANKPKMAV-       465
                       .***. .*.*..... *. .**. .** *. .****. . .*.  ...*.*. .. ....
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Fig. 1 Structure of *dsm-1*. (a) Nucleotide sequence of *dsm-1* cDNA. The amino acid sequence of the predicted open reading frame is shown below the nucleotide sequence. The poly(A) signal sequence is indicated by the open box. (b) Hydrophobicity plot of Dsm-1 protein. The hydrophobicity plot was obtained by the computer-aided calculation based on the Kyte–Doolittle analysis. (c) Sequence alignment of Dsm-1 protein with human PAPST1 and *Drosophila* SLL. The asterisks indicate the positions of amino acids identical among all the three proteins and the dots indicate the conserved amino acids between two proteins. The membrane-spanning regions predicted by the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/welcomeE.html, Tokyo University of Agriculture and Technology, Koganei, Tokyo, Japan) are shown as numbered peaks in (b), and underlined in (c). This cDNA sequence has been deposited at the Centre for Information Biology and DNA Data Bank of Japan (DDBJ) (accession no. AB197928).

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 *Sal*I 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).

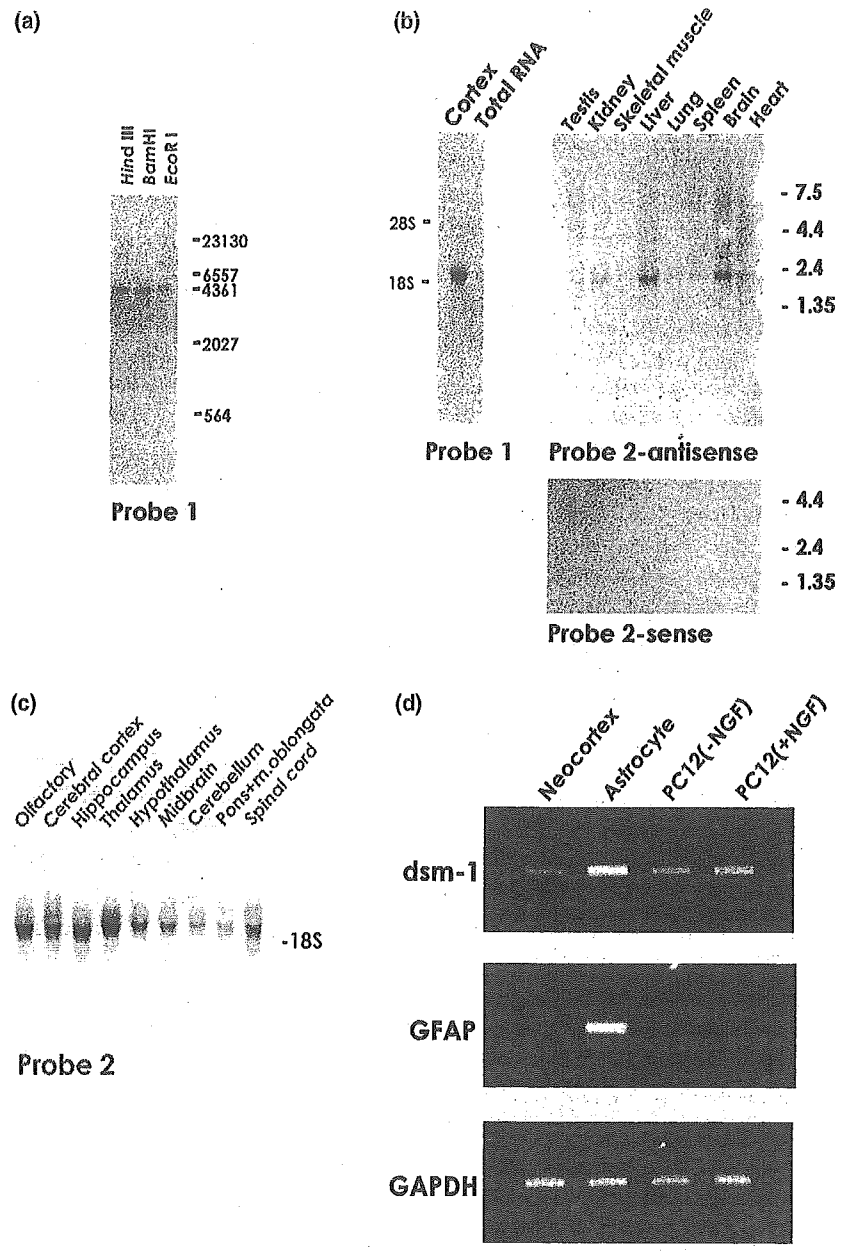

Fig. 2 Southern and northern blot, and RT-PCR analyses of *dsm-1*. (a) Southern blotting of rat genomic DNA. With a 1.3-kb probe (probe 1, see Materials and methods) for *dsm-1*, a single band at 4.1, 3.9 and 4.2 kb is detected in the rat genomic DNA digested with *Hin*dIII, *Bam*HI and *Eco*RI, respectively. (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

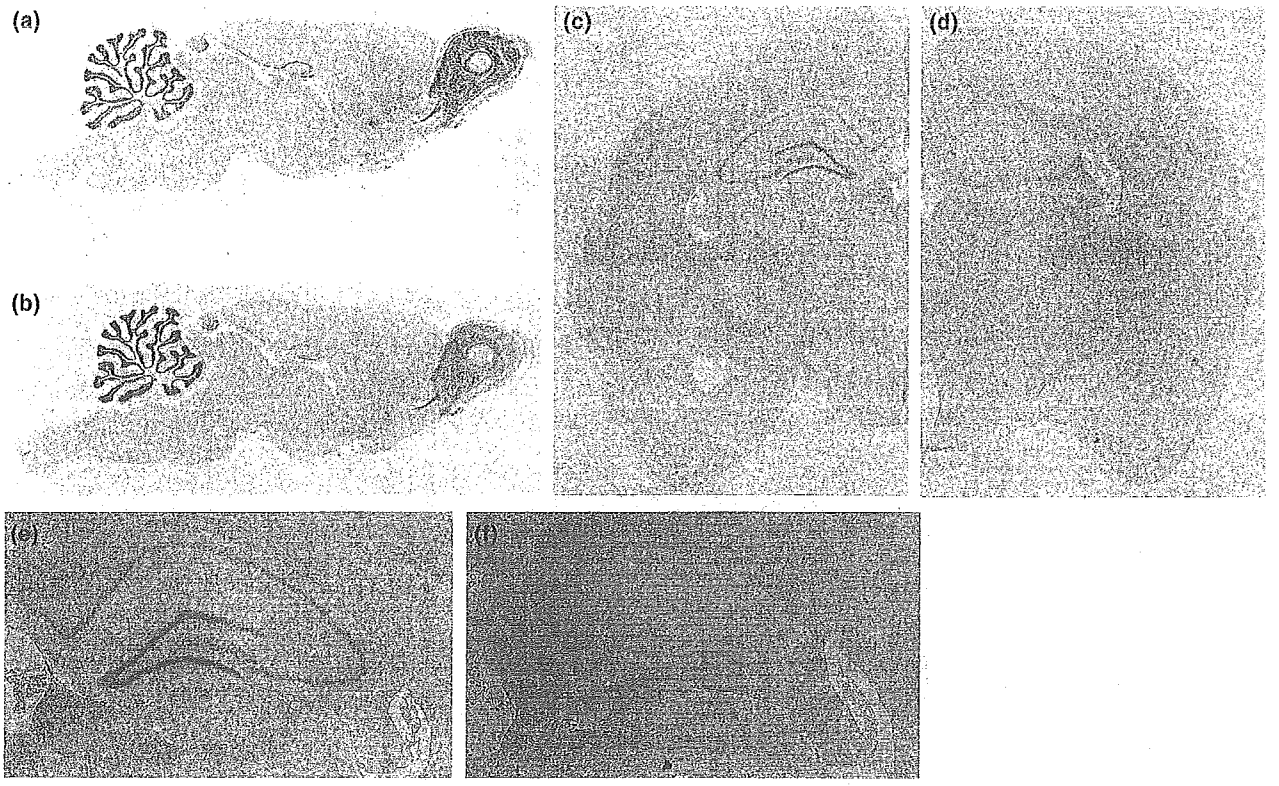

Fig. 3 *In situ* hybridization histochemistry of *dsm-1* in rat brain. The sagittal section (a, b) and the coronal section at hippocampus level (bregma −3.30 mm) (c–f) of the rat brain were hybridized with the *dsm-1* antisense (a, c, e) and sense (b, d, f) strands of probe 2. The RNA signals are detected 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

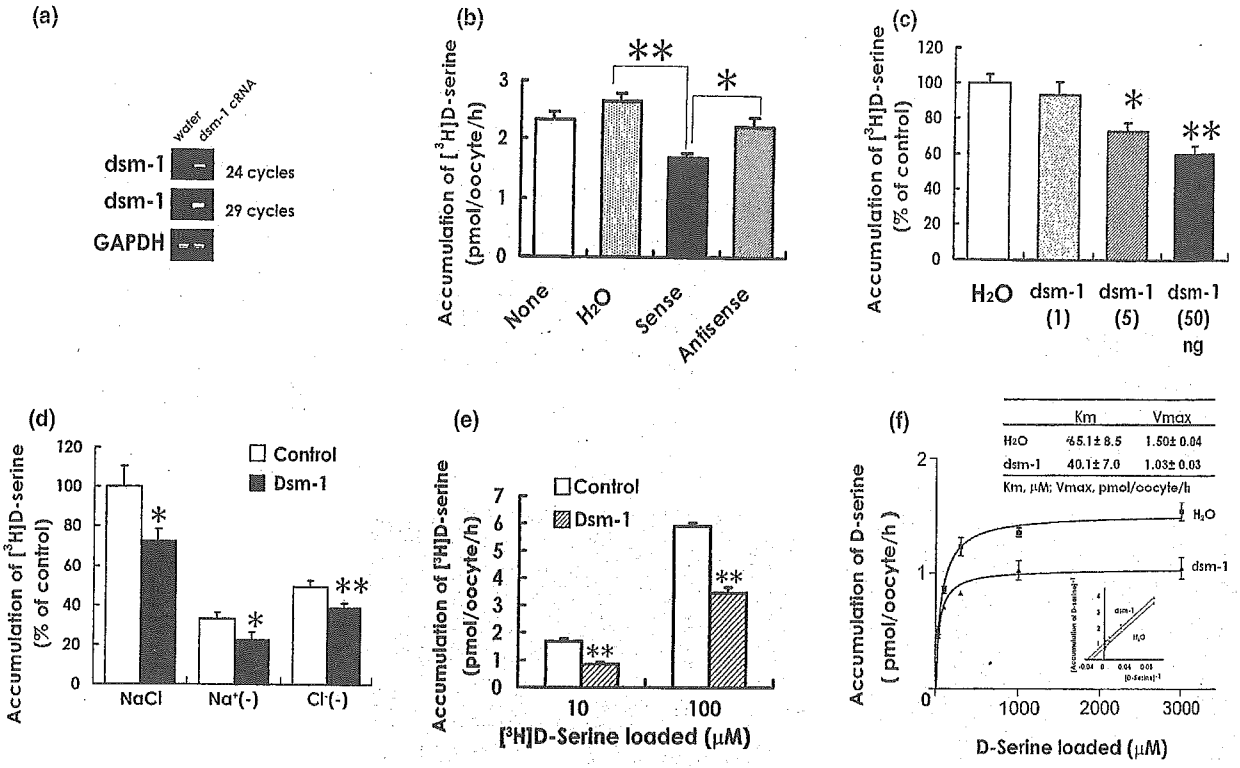

Fig. 4 Modification by Dsm-1 expression of [^{3}H]D-serine accumulation in the *Xenopus* oocytes. (a) Detection of *dsm-1* in *Xenopus* oocytes after the injection of cRNA. RT-PCR was performed to confirm that the *dsm-1* transcript was detectable at the time point of the D-serine uptake assay 2 days 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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]D-serine efflux that was calculated as the percentage of the total pre-loaded [^{3}H]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 [^{3}H]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 [^{3}H]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).

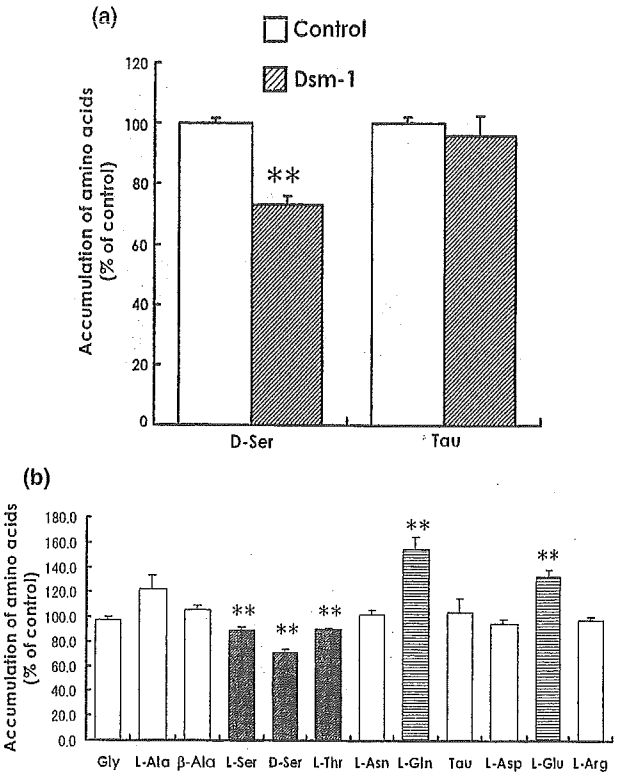

Fig. 5 Effect of Dsm-1 expression on the accumulation of various amino acids in the *Xenopus* oocytes. A, Effect of Dsm-1 on the accumulation of D-serine and taurine. The accumulation of D-serine and taurine, individually loaded at 100 μM, were, respectively, measured by HPLC. The oocytes expressing *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

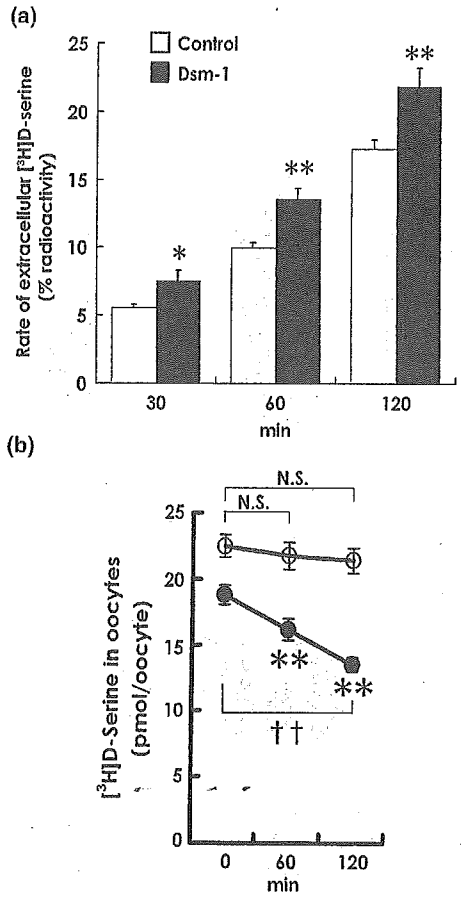

Fig. 6 Effect of Dsm-1 expression on the rate of [^{3}H]D-serine efflux from the *Xenopus* oocytes. Oocytes expressing *dsm-1* (black column and circles) and control oocytes injected with water (open column and circles) were pre-loaded with [^{3}H]D-serine for 60 min. The efflux of the pre-loaded [^{3}H]D-serine 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. $\dagger\dagger p < 0.01$ as compared with the D-serine pre-loaded control.

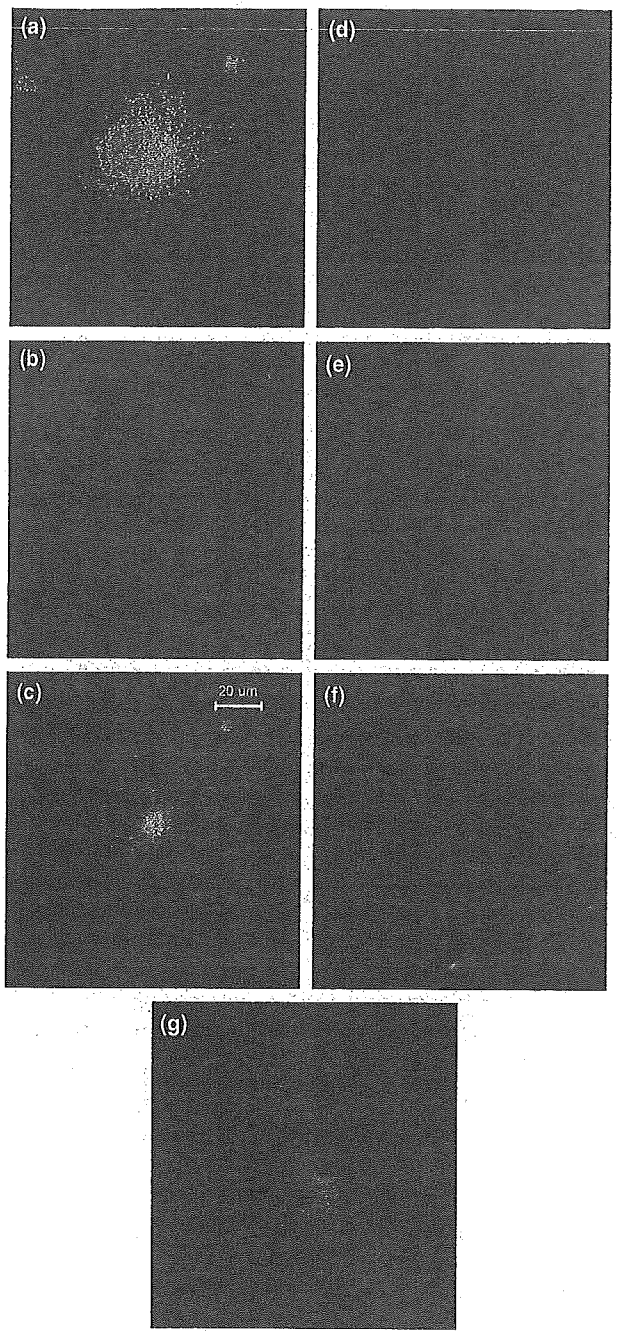

Fig. 7 Subcellular localization of Dsm-1 protein expressed in COS-7 cells. COS-7 cells were transfected with the Lumio-V5-tagged *dsm-1* fusion construct (a, b, c, g) or vector alone as a control (d, e, f). The Dsm-1 fusion protein was observed by the immunofluorescence detection with the anti-V5 monoclonal 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 *PAPSTI* 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.

