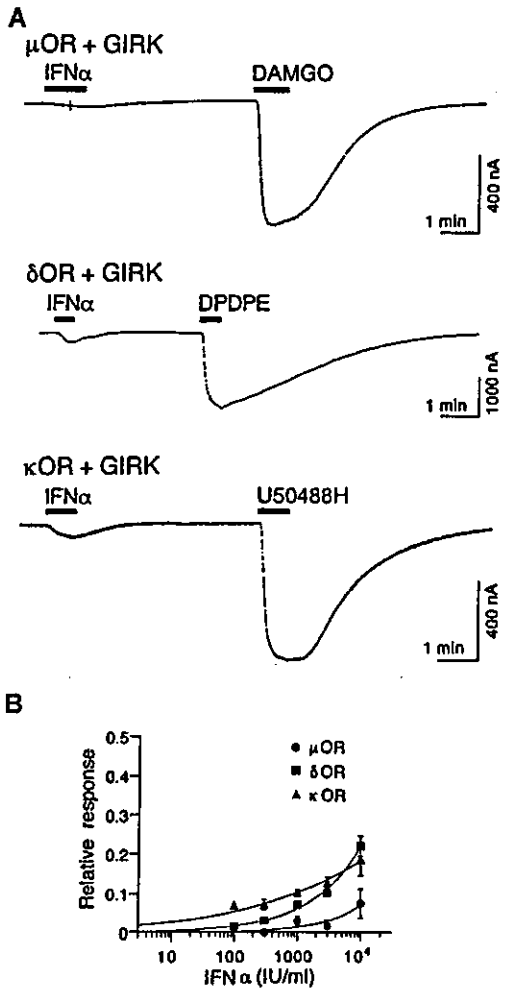

Fig. 1. Effects of IFNα on the cloned μ-, δ- and κ-opioid receptors. (A) Top: Current responses in an oocyte co-injected with the μ-opioid receptor (μOR) and GIRK1/GIRK2 mRNAs to 3000 IU/ml IFNα and to 300 nM DAMGO. Middle: Current responses in an oocyte co-injected with the δ-opioid receptor (δOR) and GIRK1/GIRK2 mRNAs to 3000 IU/ml IFNα and to 400 nM DPDPE. Bottom: Current responses in an oocyte co-injected with the κ-opioid receptor (κOR) and GIRK1/GIRK2 mRNAs to 3000 IU/ml IFNα and to 500 nM U50488H. Current responses were measured at a −70 mV membrane potential in a high-potassium solution including 96 mM K^+. Bars above the traces show the duration of application. (B) Concentration-response relationships for the effects of IFNα on the three subtypes of opioid receptors. The relative responses are the ratios of IFNα-induced responses to the control response to each of the μ-, δ- and κ-selective opioid receptor agonists, which are 300 nM DAMGO (n = 7), 400 nM DPDPE (n = 4) and 500 nM U50488H (n = 7), respectively. Each point and error bar represents the mean and S.E.M. of the relative responses.

concentration-dependent, when compared with the full response induced by a selective δ- or κ-opioid receptor agonist, DPDPE (1005.0 ± 121.6 nA, 400 nM, n = 4) or U50488H (976.8 ± 244.2 nA, 500 nM, n = 7), respectively (Fig. 1B). In contrast, in oocytes co-injected with μ-opioid receptor mRNA and GIRK1/GIRK2 mRNAs, application of IFNα, even at 3000 or 10000 IU/ml, produced little or no

current response, although 300 nM DAMGO, a selective μ-opioid receptor agonist, induced GIRK currents (668.5 ± 91.8 nA, $n = 7$, Fig. 1). Furthermore, to determine whether IFNα has antagonistic or other modulation effects on μ-, δ- and κ-opioid receptors, we examined opioid receptor activation by opioid agonists in the presence of IFNα. In oocytes expressing each of opioid receptors and GIRK1/2 channels, current responses to each selective opioid agonist together with IFNα at 100 or 1000 IU/ml were not significantly different from responses to each opioid agonist alone ($P > 0.05$, paired t-test, $n = 3$ for each, Fig. 2), suggesting that IFNα has no obvious antagonist or potentiation effect on the opioid receptors.

Heterodimerization of μ- and δ-opioid receptors or of δ- and κ-opioid receptors has been shown to occur in heterologous cell expression systems, whereas cell-membrane expression of μ- and κ-opioid receptor complexes has not been confirmed yet (Devi, 2001; Levac et al., 2002). We next investigated whether IFNα can interact with heteromers of the opioid receptors by using oocytes co-injected with mRNAs for the μ/δ-, δ/κ- or μ/κ-opioid receptors together with GIRK1/GIRK2 mRNAs. As shown in Fig. 3, current responses to 3000 IU/ml IFNα were much smaller than those to selective opioid receptor agonists ($n \geq 3$). The ratios of the responses to IFNα to the control responses to selective opioid agonists in these injected oocytes were also smaller than those in oocytes expressing either δ- or κ-opioid receptors and GIRK channels ($P < 0.05$, Student's t-test), suggesting some type of association between opioid receptors, probably partial existence of the heteromeric opioid receptors. These results

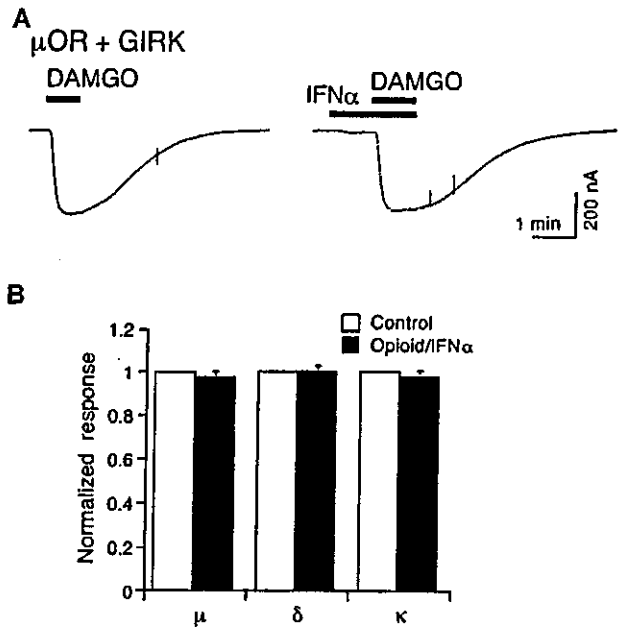

Fig. 2. Lack of antagonism of IFNα toward the cloned μ-, δ- and κ-opioid receptors. (A) Representative current responses in an oocyte co-injected with the μ-opioid receptor (μOR) and GIRK1/GIRK2 mRNAs to 200 nM DAMGO and to 200 nM DAMGO in the presence of 1000 IU/ml IFNα. Current responses were measured at a −70 mV membrane potential in a high-potassium solution including 96 mM K^+. Bars above the traces show the duration of application. (B) Effect of IFNα on activation of the three opioid receptors. The current responses to each selective opioid agonist plus 1000 IU/ml IFNα (black bars) were normalized to the control current responses (white bars) to DAMGO (200 nM), DPDPE (300 nM) or U50488H (150 nM) which were 714.2 ± 97.5 nA, 353.7 ± 29.6 nA or 1501.7 ± 75.8 nA, respectively ($n = 3$).

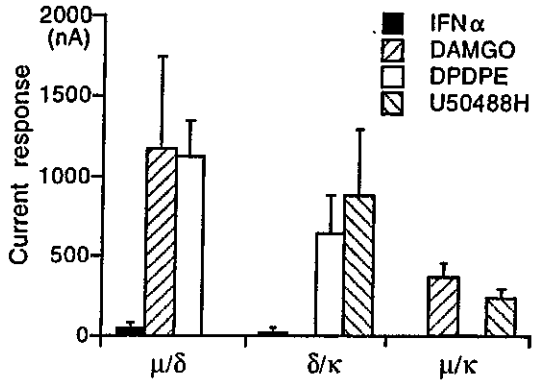

Fig. 3. Comparison of the effects of 3000 IU/ml IFNα and selective opioid receptor agonists: 300 nM DAMGO, 100 nM DPDPE and 100 nM U50488H, on oocytes expressing μ/δ-, δ/κ- or μ/κ-opioid receptors and GIRK1/2 channels ($n \geq 3$).

suggest that IFNα cannot effectively activate the heteromeric opioid receptors. In addition, current responses to selective opioid agonists were not significantly affected by the presence of 1000 IU/ml IFNα ($P > 0.05$, paired t-test, $n = 3$). Therefore, it is likely that IFNα has no obvious effect on heteromers of the receptors.

Discussion

We demonstrated that IFNα weakly activated the δ- and κ-opioid receptors, but had little effects on the μ-opioid receptor, at high concentrations, using a *Xenopus* oocyte functional assay system expressed with cloned opioid receptors and GIRK channels. Furthermore, heteromers composed of the opioid receptor subtypes were likely to be insensitive to IFNα. In IFNα therapy, the peak plasma concentrations after the systemic administration of IFNα range from approximately 100 to 750 IU/ml (Hausfater et al., 2002), although the physiological levels are constantly low (Maeyer and Maeyer-Guignard, 1998). Also, the plasma concentrations were reported to increase dose-dependently and reach 1000 IU/ml or more after high-dose administration (Shah et al., 1984). Therefore, the present results suggest that weak activation of the δ- and κ-opioid receptors by IFNα may partly contribute to some of the IFNα effects observed under the high-dose medication. However, since IFNα can not readily penetrate the blood-brain barrier (Smith et al., 1985), its CNS effects may not be mediated by opioid receptors. Because IFNα receptors which are highly sensitive to IFNα are also present in the brain (Janicki, 1992), IFNα may affect many brain functions via IFNα receptors. However, it is also possible that some IFNα metabolites may have potent opioid properties and be able to gain entry to the brain.

In animal studies, the binding of [^{3}H]naloxone to brain membranes was inhibited by IFNα at concentrations above 500 IU/ml, with IC_{50} values of 1200 IU/ml at 37 °C or approximately 3000 IU/ml (Menzies et al., 1992) or 2190 IU/ml at 25 °C (Panchenko et al., 1987). In addition, IFNα inhibited the binding of [^{3}H]DADLE, a relatively selective δ-opioid receptor agonist with moderate affinity for the μ-opioid receptor (Raynor et al., 1994), with an IC_{50} value of 1250 IU/ml at 25 °C (Panchenko et al., 1987). It also inhibited the binding of [^{3}H]dihydromorphine, which binds to the μ-opioid receptor with high affinity as well as to the δ- and κ-opioid receptors with low affinity (Schmidt et al., 2002), with an

IC_{50} value of 5000 IU/ml at 37 °C (Blalock and Smith, 1981). The results of these binding assays suggest that IFNα may temperature-dependently bind to opioid receptors at rather high levels in the clinically relevant concentrations. Although IFNα weakly activated the cloned δ- and κ-opioid receptors at 20 °C in the present study using *Xenopus* oocyte functional assays which are generally carried out at approximately 20 to 25 °C, it is possible that IFNα may efficaciously act at the opioid receptors at 37 °C in other functional assay systems.

IFNα induces various naloxone-sensitive CNS effects, namely, opioid-like effects including analgesia and catalepsy (Blalock and Smith, 1981) and modulation of wet-dog shakes induced by a $5HT_2$ receptor agonist (Kugaya et al., 1996), EEG activity (Birmanns et al., 1990; De Sarro et al., 1990), and neuronal activity in several brain regions at concentrations of less than 5000 IU/ml (Nakashima et al., 1987, 1988). Additionally, among the IFNα-induced effects, its analgesic effect in a tail-flick test (Jiang et al., 2000), an increase in immobility in a forced swimming test (Makino et al., 2000) and suppression of hypothalamo-pituitary-adrenocortical secretory activity (Saphier et al., 1993) were inhibited by a selective μ-opioid receptor antagonist, but not by selective δ- and κ-opioid receptor antagonists. Our results fail to support the theory that the IFNα effects are mediated by the direct action of IFNα on the μ-opioid receptor. However, weak δ- and κ-agonist properties of IFNα in the present study may partly contribute to some of the naloxone-sensitive effects. We suggest that most of the CNS effects induced by IFNα may be mainly mediated by IFNα receptors in the brain, resulting in functional enhancement of the opioid systems. On the other hand, IFNα caused the opposite effects of morphine, a relatively selective μ-opioid receptor agonist with low affinity for the κ-opioid receptor (Raynor et al., 1994), in guinea-pig ileum preparations which are used in bioassays for characterizing activation of the μ- and κ-opioid receptors (Leslie, 1987) and on the activity of cortical neurons, acting in a naloxone-insensitive manner (Reyes-Vazquez et al., 1984). These observations suggest that the IFNα actions may not be mediated by opioid receptors. The discrepancy between these earlier findings and ours might be explained by the difference in the methods between the microiontophoretic application and perfusion application of IFNα and by the difference in properties of the preparations used. Namely, in the case of the microiontophoretic application, it is difficult to determine the precise concentrations in experimental preparations. The present study showed that IFNα at high concentrations slightly activated the cloned δ- and κ-opioid receptors. Taken together, microiontophoretically applied IFNα might have no significant effect on opioid receptors at concentrations of IFNα in their experimental preparations, and the naloxone-insensitive IFNα effects might be mediated mainly by IFNα receptors.

IFNα inhibited cAMP accumulation by forskolin in SH-SY5Y cells expressing μ-opioid receptors predominantly and δ-opioid receptors, but not in NG 108-15 cells expressing δ-opioid receptors, and the inhibitory effects were prevented by naloxone (Saphier et al., 1994; Jiang et al., 2000), suggesting activation of the μ-opioid receptor by IFNα. However, such results are inconsistent with our present results on the μ-, δ- or κ-opioid receptors expressed in *Xenopus* oocytes. In addition, this study suggests that IFNα is unlikely to have significant effects on the μ/δ-opioid heteromeric receptors. Because the existence of multiple pharmacologically defined opioid receptor subtypes has been suggested (Levac et al., 2002), novel opioid receptor genes might be found. Recent studies have shown heteromerization between opioid receptors and other G-protein-coupled receptors (GPCRs), such as $somatostatin_{2b}$ receptors and β_2-adrenoceptors, as well as opioid receptor heteromerization, and some of the heteromeric receptors exhibit novel pharmacological characteristics (Devi, 2001; Levac et al., 2002; Pfeiffer et al., 2002). Therefore, it is possible that novel opioid receptors or heteromers of opioid receptors and other GPCR(s) or of other members of GPCRs in the clonal cells might exhibit the

pharmacological characteristics of the opioid receptors sensitive to IFNα and naloxone. In addition, in some types of cells co-expressing IFNα receptors and opioid receptors, IFNα receptor-mediated signal transduction might cause functional enhancement of opioid receptors. Therefore, the physiological and pharmacological effects of IFNα may be mediated by not only IFNα receptors but also the δ- and κ-opioid receptors and such GPCRs.

Conclusion

The present study demonstrates that IFNα directly and weakly activates the δ- and κ-opioid receptors. The activation by IFNα may partly contribute to some of the effects under its high-dose medication.

Acknowledgments

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Functional identification of ASCT1 neutral amino acid transporter as the predominant system for the uptake of L-serine in rat neurons in primary culture☆

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Abstract

The uptake of L-serine, a nonessential amino acid known to be transported by the neutral amino acid transporter system ASC, was studied in primary cultures of rat neurons and astrocytes, and compared with that in human embryonic kidney (HEK293) cells transfected with rat ASCT1 cDNA. We first cloned neutral amino acid transporter ASCT1 from rat neurons in primary culture as a transporter candidate for L-serine uptake in the brain. The predicted amino acid sequence from rat ASCT1 exhibited significant homology with mouse and human ASCT1s. The amino acid sequence of rat ASCT1 was 92 and 84% identical to that of mouse and of human ASCT1, respectively. HEK293 cells expressing the rat ASCT1 cDNA showed a saturable dose-dependent and Na^+-dependent increase in L-[^{3}H] serine uptake by high affinity (K_m = 67 μM). The substrate selectivity of rat ASCT1 was the same as those of the mouse and human transporter. Northern blot analysis revealed that ASCT1 mRNA was ubiquitously expressed in the brain, with its highest concentration in the striatum and hippocampus. When the uptake of L-[^{3}H] serine into rat primary neurons or astrocytes was compared with that of HEK293 cells expressing rat ASCT1 or rat ASCT2 cDNA, the inhibition profile of amino acids for the rat neurons quite resembled that for HEK293 cells expressing rat ASCT1. In contrast, the profile for rat astrocytes was a mixture of that for HEK293 cells expressing rat ASCT1 and that for the cells expressing rat ASCT2. Furthermore, L-[^{3}H] serine uptake in neurons was fully Na^+-dependent. ASCT1 mRNA was expressed in both primary neurons and astrocytes, whereas ASCT2 mRNA was expressed only in astrocytes, as determined by using RT-PCR with primers specific for the rat ASCT1 or rat ASCT2 transporter. Taken together, these findings indicate that ASCT1 predominantly contributes to the uptake of L-serine in primary neurons.

Keywords: ASCT1; ASCT2; cDNA cloning; Functional assay; Northern blot; Brain regional distribution

1. Introduction

L-Serine is a nonessential amino acid that can be synthesized in many cells from the glycolytic pathway. Previously, it was suggested that cerebellar Purkinje neurons had an essential requirement for extracellular L-serine to support their survival (Fuyuya et al., 2000). Studies using immunohistochemistry and in situ hybridization indicated that cerebellar Purkinje neurons showed no detectable mRNA

Abbreviations: 3PGDH, 3-phosphoglycerate dehydrogenase; MeAIB, 2-methylaminoisobutylate; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; GAPDH, glyceraldehydes-3-phosphate dehydrogenase

☆ The nucleotide sequence reported in this paper has been submitted to GenBank as accession number AB103401.

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or immunoreactivity for 3-phosphoglycerate dehydrogenase (3PGDH; Fuyuya et al., 2000; Yamazaki et al., 2001), which is the enzyme-initiating L-serine synthesis from the glycolytic intermediate 3-phosphoglycerate. Therefore, specific transport mechanisms are necessary to provide such 3PGDH-negative neurons with L-serine from the extracellular space to support their survival and development.

L-Serine is a key amino acid serving as a precursor for not only the biosynthesis of various protein and membrane lipid molecules, but also for the synthesis of the neurotransmitters glycine and D-serine in the brain (Wolosker et al., 1999a,b), which are thought to be especially important by acting at the "glycine site" on the *N*-methyl-D-aspartate (NMDA) receptor complex to regulate its ion channel functions in the central nervous system (CNS; Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Kemp and Leeson, 1993; Matsui et al., 1995; Ivanovic et al., 1998).

Neutral amino acid L-serine can be taken up through several transport systems, including Na^+-dependent transporters such as system ASC and system A, and Na^+-independent transporters, e.g., system L and system ASC. Being system ASC transporters, ASCT1 and ASCT2 were identified and their cDNAs were cloned from human and mouse (Arriza et al., 1993; Shafqat et al., 1993; Kekuda et al., 1996; Utsunomiya-Tate et al., 1996). Although the expression of the ASCT1, but not ASCT2, subtype of neutral amino acid transporter has been detected in the brain, detailed aspects of and its roles in L-serine transport in the brain are not yet fully understood. In the present study, we cloned rat ASCT1 cDNA and expressed the protein encoded by its cDNA for functional characterization to clarify the contribution of the system ASC transporters to L-serine uptake in neurons and astrocytes. We confirmed the previous hypothesis (Yamamoto et al., 2003) that L-serine is transported predominantly through the ASCT1 transporter subtype in neuron.

2. Materials and methods

2.1. Materials

L-[^{3}H] serine (specific radioactivity: 851.0 GBq/mmol) was purchased from Perkin-Elmer Life Sciences, and [α-^{32}P] dCTP was obtained from Amersham Pharmacia Biotec. 2-Methylaminoisobutyrate (MeAIB), 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), and all amino acids were obtained from Sigma-Aldrich. All other reagents used were of analytical grade.

2.2. Cell culture

Primary cultures of neurons and astrocytes were prepared from fetal rat telencephalon (embryonic day 18 or 19) as described before (Yamamoto et al., 1995). Pregnant rats were deeply anesthetized with pentobarbital (50 mg/kg, i.p.). Tissues were incubated for 15–20 min at 37 °C with 0.25% trypsin (Difco) in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (CMF-PBS) containing 0.02% DnaseI (Sigma), dissociated by mechanical dispersion by repetitive pipetting and filtered through a nylon mesh. For neuronal culture, the cells were rinsed with defined culture medium, Dulbecco's-modified Eagle's medium (DMEM), and plated on poly-L-lysine-coated 96-well plates at a final density of (0.5–1.5) × 10^6 cells/ml. The cultures were maintained for 7 days in DMEM supplemented with 1 mg/ml bovine serum albumin (BSA), 10 μg/ml insulin, 1 nM 3,3′,5-triiodo-L-thyronine, 0.1 mg/ml human transferrin, 1 μg/ml aprotinin, 100 μM putrescine, 1 mM sodium pyruvate, 10 nM progesterone, 30 nM selenium, 0.1 mg/ml streptomycin sulfate, and 50 units/ml penicillin G potassium salt (Meiji Seika Ltd., Tokyo, Japan) in a humidified atmosphere of 5% CO_2 in air at 37 °C. More than 95% of the cultured cells were immunonegative when stained with anti-glial fibrillary acidic protein (GFAP) antibody (data not shown). Cultures of astrocytes were obtained by starting with the same preparation of dissociated cells described above used for preparing neuronal cultures. The dissociated cells were subcultured in DMEM containing 5% fetal calf serum (FCS) and 5% horse serum (HS) supplemented with 0.1 mg/ml streptomycin sulfate, 50 units/ml penicillin G potassium salt before being plated on poly-L-lysine-coated wells of 96-well plates. The cells were maintained for 14–21 days. More than 95% of these cultured cells were immunopositive with anti-GFAP antibody (data not shown).

2.3. cDNA cloning of ASCT1 and sequence analysis

Rat ASCT1 cDNA was cloned from rat primary neuron total RNA by using the reverse transcription-based polymerase chain reaction (RT-PCR) followed by PCR with nesting primers. The two sets of degenerate oligonucleotide sense and antisense primers used were ASCT-5 (ACGACATGGAGAAGAG(C/T)GG(A/G/C/T)GA(A/G)AC(A/G/C/T)AA)/ASCT-4 (CATGTTGACGGT(A/G/C/T)GC(A/G/C/T)CC(A/G/T)AT) and ASCT-1 (ATGATCATCCTGCC(A/G/C/T)CT(A/G/C/T)GT(A/G/C/T)G)/ASCT-6 (AATCACAGCAC(C/T)GA(C/T)TC(C/T)TT(A/G/C/T)GA(C/T)TC), which corresponded to amino acid residues 1–8/373–379 and 88–95/526–532 of human ASCT1 (Arriza et al., 1993). The three sets of primers used for the nested PCR were ASCT-5/ASCT-3 (ATAATCCAGGA(A/G/C/T)AC(A/G/C/T)AG(A/G/C/T)ACCAT), ASCT-2 (GGTCCTGGT(A/G/C/T)TC(A/G/C/T)TGGAT(A/C/T)ATG)/ASCT-6, and ASCT-7 (AGACGAGCTCATCCGATTCTTC)/ASCT-4, which corresponded to amino acid residues 1–8/259–266, 259–266/526–532, and 244–251/373–379, respectively. PCRs were carried out for 0.5 min at 94 °C, 0.5 min at 55 °C, and 2 min at 72 °C for 40 cycles. The 800, 830, and 410 bp products amplified by the nested PCR were blunted with T4 DNA polymerase and cloned into the *Stu*I site of pCR-Blunt (Invitrogen) to yield

plasmids pASCT-1P, pASCT-2P, and pASCT-3P, respectively. The 190 bp *SacI/NcoI* fragment from pASCT-3P and the 680 bp *NcoI/Eco*RI fragment from pASCT-2P were ligated with the 2.9 kb *SacI/Eco*RI fragment from pBluescript SK(+) (Stratagene) to yield pBlASCT-4P. The 880 bp *SacI/Hin*dIII fragment from pBlASCT-4P was ligated with the 4.2 kb *SacI/Hin*dIII fragment from pASCT-1P to yield pASCT1. Nucleotide sequence analysis was performed using an ABI PRISM 377 and 373A automated DNA sequencers (Perkin-Elmer Life Sciences). The 3′ end of rat ASCT1 cDNA was obtained by 3′ rapid amplification of cDNA ends (3′-RACE), which was performed using a 3′ full RACE Core Set (TaKaRa, Japan) according to the manufacturer's instructions. PCR amplification was performed using a rat ASCT1-specific forward primer (5′-GGACTGCCCACGAACGACCTCTC-3′), which corresponded to amino acid residues 438–446, and an oligo(dT)-adaptor primer in the kit. PCR amplification of the 5′ region of rat ASCT1 cDNA was carried out using a primer pair 5′-CAGGAACATGATGCCGATAGGTA-3′ (antisense), which corresponded to amino acid residues 266–274, and 5′-AGTTTCTCACGTGCTGCTGCTTAG-3′ (sense), which was designed on the basis of the rat genome library (nucleotides: 165–188; GenBank accession number: XM223649). BLAST programs were used to search for homologies in protein data banks.

ASCT2 cDNA containing full open reading frame was cloned by using RT-PCR from total RNA of rat primary cerebellar mixed culture (Furuya et al., 1998) with oligonucleotide primers MDAS2F1 (sense, 5′-CACAAGGAACCTCCCTGTTACGGCT-3′) and MDAS2R1 (antisense, 5′-AGTCCATTTCTCCAGCTCGCAAGAC-3′), which correspond to 466–490 and 2206–2230 of mouse ASCT2 cDNA, respectively (GenBank accession number: D85044; Utsunomiya-Tate et al., 1996). PCR conditions: denaturing at 94 °C for 0.5 min, annealing/extension at 66 °C for 4 min. PCR products were directly ligated into pCRII-TOPO vector (Invitrogen), purified, and sequenced. The amino acid sequence of the rat cerebellar homologue of ASCT2 is identical to the rat astroglial ASCT2 except for S-529 to P (Broer et al., 1999; Furuya et al., unpublished observations). The rat cerebellar ASCT2 was excised by *Kpn*I and *Eco*RV digestions and inserted into the *Kpn*I/*Eco*RV sites of pcDNA3.1(+) to yield pCDASCT2.

2.4. Expression in HEK293 cells

The 1.6 kb blunted *Eco*RI fragment containing the entire coding region of the rat ASCT1 or ASCT2 was inserted into the *Eco*RV site of pcDNA3.1(+) (Invitrogen) in the same orientation with respect to gene transcription, to yield expression vector, pCDASCT1 or pCDASCT2. HEK293 cells were grown in DMEM supplemented with 5% FCS, penicillin G, and streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO_2 in air. Transfection by lipofectamine reagent (Invitrogen) was carried out according to the manufacture's instructions. Transformants were selected for 2–3 weeks in culture medium supplemented with 2.0 mg/ml G418. Among G418-resistant transformants, ASCT-transformed HEK293 clones were selected by L-[^{3}H] serine uptake analysis.

2.5. L-[^{3}H] serine uptake experiment

On the day of an experiment, the culture medium was aspirated from HEK293 cell or rat neuronal or astrocyte cell cultures, and these cells were washed three times with 0.1 ml of uptake buffer containing 5 mM HEPES NaOH (or KOH), pH 7.2, 140 mM NaCl (or choline-Cl), 5 mM KCl, 1 mM KH_2PO_4, 1.8 mM $CaCl_2$, 0.4 mM $MgCl_2$, and 10 mM glucose. After the cells had been preincubated in the uptake buffer for 30 min at 37 °C, they were incubated at 37 °C for 5 min in 0.1 ml of fresh uptake buffer supplemented with 20 nM (final concentration) L-[^{3}H] serine and different amounts of unlabeled amino acids. Uptake was terminated by rapid removal of the medium followed by quick washes with ice-cold phosphate-buffered saline (PBS). To measure the amount of L-[^{3}H] serine taken up into cells, we added 50 μl of a 10% SDS solution to each well and solubilized the cells for 1 h at 37 °C. The radioactivities were measured by using a liquid scintillation counter. Each uptake was represented as the mean of 3–5 independent experiments, each done in quadruplicate. Na^+-independent uptake was carried out by incubation of cells in medium containing choline chloride instead of NaCl. For inhibition experiments by various amino acids, the concentrations of L-[^{3}H] serine and amino acids (20 nM and 0.5 mM, respectively) were chosen to detect high-affinity uptake site according to the K_m values (15–17 μM) for L-[^{3}H] serine uptake to cultured neurons and astrocytes (Yamamoto et al., 2003). Protein content was measured by using a Bradford protein assay kit (Bio-Rad, Richmond, CA) after the cells had been solubilized with 0.1 M NaOH.

2.6. RT-PCR analysis

Expression of ASCT1 and ASCT2 transcripts in primary cultures of rat neurons and astrocytes, HEK293 cells, and in vector-, ASCT1-, and ASCT2-transfected HEK293 cells was examined by RT-PCR. Total RNA was isolated from the cultured cells by using a Catrimox-14 RNA Isolation kit (TaKaRa, Japan) according to the manufacturer's protocol. RT-PCR was performed with an RNA LA PCR kit (TaKaRa, Japan). RT was performed at 50 °C for 30 min, followed by a denaturation at 90 °C for 5 min. PCR amplification of cDNAs of ASCT1, ASCT2, and GAPDH (as an internal standard) was performed with 35 cycles of 94 °C for 0.5 min for denaturation, 58 °C for 0.5 min for primer annealing, and 72 °C for 2 min for DNA extension. Sequences of the primers for ASCT1, ASCT2, and GAPDH cDNAs were the following: 5′-GTTTGCGACGGCTTTTGCGACCTG-3′ (forward primer) and 5′-GCATCCCCTTCCACGTTCACCACA-3′ (reverse primer) for rat ASCT1(product size: 399 bp;

GenBank accession number: AB103401); 5′-GCGCCTGGGCCCTGCTCTTTTT-3′ (forward) and 5′-ACAATCTTGCCGGCCACCAGGAAC-3′ (reverse) for rat ASCT2 (product size: 478 bp; GenBank accession number: AJ132846); 5′-GTCAGTGCCGGCCTCGTCTCATAG-3′ (forward) and 5′-GACCCTTTTGGCACCACCCTTCAG-3′ (reverse) for rat GAPDH (product size: 380 bp; GenBank accession number: M17701). The PCR amplification products were separated on a 3% agarose gel stained with ethidium bromide and visualized under UV irradiation.

2.7. Northern blot analysis

For Northern blot analysis, total RNA from various regions of rat brain was isolated by using an RNeasy Midi kit (Qiagen Inc.). Equal amounts of total RNA (40 μg) were separated on a 1.0% agarose gel containing formaldehyde and transferred to a nitrocellulose membrane (BA85, S&S) by using a capillary blotting technique with 20× SSC (sodium citrate buffer). Fragments of rat ASCT1 and GAPDH cDNAs were amplified by PCR and purified by use of a Qiagen

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GGCGGGCAGCGACATGGAGGAGACCAACGGATACCTCGACGGCGCCCAGGCTCAGCCTGCGGCCCGTCCCCGA      60
                M  E  E  T  N  G  Y  L  D  G  A  Q  A  Q  P  A  A  R  P  R
ACGCCCGAGACGGGGACGAGCAAAGCGCAGCGCTGTGCTAGCTTTTTTCGGCGCAACGCGCTGGTGCTGCTCACC      335
 T  P  E  T  G  T  S  K  A  Q  R  C  A  S  F  F  R  R  N  A  L  V  L  L  T
GTGTCGGGGGTGTTGGCGGGCGCCGGCATGGGCGCGGCGCTTCGCGGGCTGCAGCTCACCGCACGCAAATCACT      210
 V  S  G  V  L  A  G  A  G  M  G  A  A  L  R  G  L  Q  L  T  R  T  Q  I  T
TACCTGGCCTTCCCGGGCGAGATGCTGCTCCGTATGCTGCGCATGATCATCCTACCGCTGGTGGTTTGCAGTCTG      285
 Y  L  A  F  P  G  E  M  L  L  R  M  L  R  M  I  I  L  P  L  V  V  C  S  L
GTGTCGGGCGCCGCCTCCCTGGACGCCAGCTCCCTCGGGCGTCTGGGTGGCATCGCTGTTGCTTACTTCGGCCTC      360
 V  S  G  A  A  S  L  D  A  S  S  L  G  R  L  G  G  I  A  V  A  Y  F  G  L
ACCACGCTGAGTGCCTCTGCGCTCGCTGTTGCTCTGGCGTTCATCATCAAGCCAGGAGTGGGCGCGCAGACCCTT      435
 T  T  L  S  A  S  A  L  A  V  A  L  A  F  I  I  K  P  G  V  G  A  Q  T  L
CATTCCAGCAGCCTGGGGCTGGAGAACTCAGAACCTCCTCCAGTCTCCAAAGAGACAGTGGACTCTTTCCTGGAT      510
 H  S  S  S  L  G  L  E  N  S  E  P  P  P  V  S  K  E  T  V  D  S  F  L  D
TTACTCAGAAACCTGTTCCCTTCCAATCTTGTGGTTGCCGCATTCTCTACGTCTGCAACCAGTTACACAGTGGTC      585
 L  L  R  N  L  F  P  S  N  L  V  V  A  A  F  S  T  S  A  T  S  Y  T  V  V
GTCCATAACTCCAGCTTGGGGAACGTGACCAAAGAGAAGATCCCCGTCGTCACTGATGTGAAAGGGATGAACATC      660
 V  H  N  S  S  L  G  N  V  T  K  E  K  I  P  V  V  T  D  V  K  G  M  N  I
TTAGGACTGGTCCTTTTTGCCCTGATGTTAGGAGTGGCTCTAAAGAAGCTAGGCCCCGAGGGAGACGAGCTCATC      735
 L  G  L  V  L  F  A  L  M  L  G  V  A  L  K  K  L  G  P  E  G  D  E  L  I
CGATTCTTCAATTCCTTCAATGAGGCAACAATGGTGTTGGTGTCGTGGATCATGTGGTACGTACCTATCGGCATC      810
 R  F  F  N  S  F  N  E  A  T  M  V  L  V  S  W  I  M  W  Y  V  P  I  G  I
ATGTTCCTGATCGGAAGCAAGATTGTGGAAATGCAGGACCTCATCGTGCTCGTGACTAGCCTCGGGAAATACATC      885
 M  F  L  I  G  S  K  I  V  E  M  Q  D  L  I  V  L  V  T  S  L  G  K  Y  I
TTCGCCTCTATACTGGGCCATGTTATCCATGGAGGAATTGTTCTGCCTCTTGTCTATTTTGCTTTTACGAGGAAA      960
 F  A  S  I  L  G  H  V  I  H  G  G  I  V  L  P  L  V  Y  F  A  F  T  R  K
AACCCGTTCACGTTCCTCCTGGGCCTCCTCACCCCGTTTGCGACGGCTTTTGCGACCTGTTCCAGCTCAGCAACC      1035
 N  P  F  T  F  L  L  G  L  L  T  P  F  A  T  A  F  A  T  C  S  S  S  A  T
CTTCCGTCTATGATGAAGTGCATCGAGGAAAACAATGGCGTGGACAAGAGGATCAGCAGGTTCATCCTTCCCATC      1110
 L  P  S  M  M  K  C  I  E  E  N  N  G  V  D  K  R  I  S  R  F  I  L  P  I
GGGGCCACAGTCAACATGGATGGGGCCGCCATCTTCCAGTGTGTGGCTGCAGTGTTCATCGCCCAGCTCAACAAC      1185
 G  A  T  V  N  M  D  G  A  A  I  F  Q  C  V  A  A  V  F  I  A  Q  L  N  N
GTGGACCTGAACGCGGGACAGATTTTCACCATTCTGGTGACCGCCACCGCATCCAGTGTTGGAGCAGCGGGTGTG      1260
 V  D  L  N  A  G  Q  I  F  T  I  L  V  T  A  T  A  S  S  V  G  A  A  G  V
CCGGCTGGAGGGGTCCTCACCATTGCCATCATCCTAGAGGCCATTGGACTGCCCACGAACGACCTCTCTCTGATC      1335
 P  A  G  G  V  L  T  I  A  I  I  L  E  A  I  G  L  P  T  N  D  L  S  L  I
CTGGCTGTGGACTGGATTGTGGACAGGACCACCACTGTGGTGAACGTGGAAGGGGATGCCCTGGGAGCTGGGATC      1410
 L  A  V  D  W  I  V  D  R  T  T  T  V  V  N  V  E  G  D  A  L  G  A  G  I
CTCAACCACCTGAATCAGAGGACAGTGAAGAAGGGTGAGCAGGAGCTGCAAGAAGTGAAGGTGCAAGCCATTCCC      1485
 L  N  H  L  N  Q  R  T  V  K  K  G  E  Q  E  L  Q  E  V  K  V  E  A  I  P
AATTCCAAGTCTGAGGAGGAGACGTCGCCCCTGGTGACACACCAGAACCGGGCAGGCCCTGTAGCCGTTGCTCCT      1560
 N  S  K  S  E  E  E  T  S  P  L  V  T  H  Q  N  R  A  G  P  V  A  V  A  P
GAACTCGAATCCAAGGAGTCAGTGCTGTGATTGGGCTGGGTCTTGGGCCATCCTGCTAGTACCAGCGACCTACCC      1635
 E  L  E  S  K  E  S  V  L  *
TGGTAGCCTGCCCCACTGACGTGGGCATGGCCCTCACAGACTTTTCGTCTCCCAAGGAATGCGTTGGCCCAATCA      1710
CTAATCTGAGAGTGACCTCTCAGCACAGTCATGGAGCTCCCAAGCCAGGACTGCTTACCGAGGACTAGAACACTC      1785
TGACCTTTGTCTTGATCCATGTCTCAATGGAGCTGTGGATTCACCTTGACTCTGTTTGTGAACCCCCTTGAGCTG      1860
CCAGACCTCAGGGATCCCAGGTTCATGGAGGCCTGGGATAAAGGTCCGGGTTGGGGTGGGGGTGCGGATGGATTC      1935
TTCAGTGTCTCCCGGGAGCTCTGCATCTGGTGCTTTCTGGGCGAGCCA                                  1983
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Fig. 1. Nucleotide and deduced amino acid sequences of rat neuron ASCT1. The predicted amino acid sequence is shown below the nucleotide sequence. Numbers indicate the nucleotides, and potential *N*-glycosylation sites are boxed. The cDNA sequence has been submitted to the GenBank database with the accession number AB103401.

II gel extraction kit (Qiagen Inc.). These fragments were labeled with [α−^{32}P] dCTP by using a Random Primer DNA Labeling kit, Ver. 2 (TaKaRa, Japan). After prehybridization at 42 °C for 4 h in 5× SSPE buffer containing 40% formamide, 0.1% SDS, and 5× Denhardt's solution, the membranes were hybridized with [α−^{32}P] dCTP-labeled ASCT1 or GAPDH cDNA fragments, respectively, at 42 °C overnight in a 5× SSPE buffer containing 50% formamide, 0.1% SDS, 5× Denhardt's solution, 10% dextran sulfate, and 100 μg/ml salmon sperm DNA. The washing was done under highly stringent conditions: four washes with 2× SSC containing 0.1% SDS at room temperature and twice with 0.2× SSC containing 0.1% SDS at 42 °C for 15 min, and finally once for 1–2 h with 0.1× SSC containing 0.1% SDS at 50–60 °C. The filters were analyzed with a BAS3000 system (Fuji Film).

2.8. Calculations

All results were presented as the mean ± S.E.M. of 3–5 experiments, as indicated in each figure legend. Kinetic parameters were calculated by linear regression from Lineweaver-Burk plots by using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Cloning of ASCT1 from primary cultures of rat neurons

To look for members of the ASCT family, we used degenerated oligonucleotides that were designed to hybridize with relatively well-conserved regions between the cDNAs of mouse and human ASCT1 and ASCT2 (see Section 2) as a primer. RT-PCR amplifications were performed by using total RNA prepared from rat neurons in primary culture as a template. We obtained a single PCR amplification product, which sequence was similar to mouse and human ASCT1 sequences. The cDNA and deduced amino acid sequence of rat ASCT1 are shown in Fig. 1. The open reading frame encoded 595 amino acids with a predicted molecular weight of ~59 kDa. The putative extracellular domain contained conserved potential N-linked glycosylation sites. The rat ASCT1 amino acid sequence exhibited remarkable similarity to those sequences reported for mouse and human ASCT1s, showing 92 and 84% identity, respectively. The NH_2-terminal portion of rat ASCT1 had lost three amino acid residues found in the mouse and human ASCT1 transporters.

3.2. RT-PCR analysis of ASCT1 and ASCT2 in rat primary neurons, astrocytes, and HEK cells

Full-length rat ASCT1 and ASCT2 were constructed and used for stable transfection of HEK293 cells, and their expressions were assessed by RT-PCR. Rat ASCT1 and ASCT2 were detected as 399 and 478 bp bands, respectively, when HEK293 cells were separately transfected with ASCT1 and ASCT2 constructs and PCR was done with primer pairs specific for each (Fig. 2). In addition, rat-cultured astrocytes expressed both ASCT1 and ASCT2 transcripts, whereas the rat neurons only expressed the ASCT1 transcript (Fig. 3).

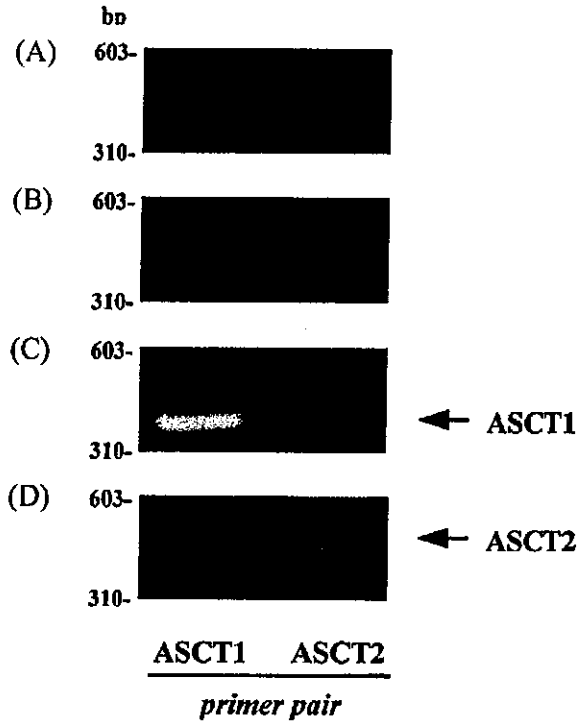

Fig. 2. Expression of rat ASCT1 and ASCT2 in HEK293 cells. Total RNA was prepared from untreated HEK293 cells (A) or those transfected with empty vector (B), ASCT1- (C), or ASCT2 cDNA (D). RT-PCR was performed to detect the expression of rat ASCT1 (left lane) or ASCT2 (right lane) by using specific primer sets as described in Section 2. PCR products were separated by electrophoresis on 3% agarose gels and stained with ethidium bromide.

3.3. L-[^{3}H] serine uptake into HEK293 cells transfected with ASCT1 or ASCT2, and into rat neurons and astrocytes in primary culture

When HEK293 cells were transfected with rat ASCT1 or ASCT2 cDNA, the uptake of L-[^{3}H] serine was increased to be seven- to nine-fold of that of the control HEK293 cells (data not shown), and this increase was totally dependent on Na^+ ions in the buffer (Fig. 4). In each case the uptake of L-[^{3}H] serine was saturable and followed Michaelis-Menten kinetics, which indicated a single uptake site with a K_m value of 67.1 ± 13.3 μM for ASCT1 and of 66.1 ± 9.0 μM (mean ± S.E.M, $N = 4$) for ASCT2 (Fig. 5).

The substrate specificity of the transporters in HEK293 cells transfected with ASCT1 or ASCT2 was examined by

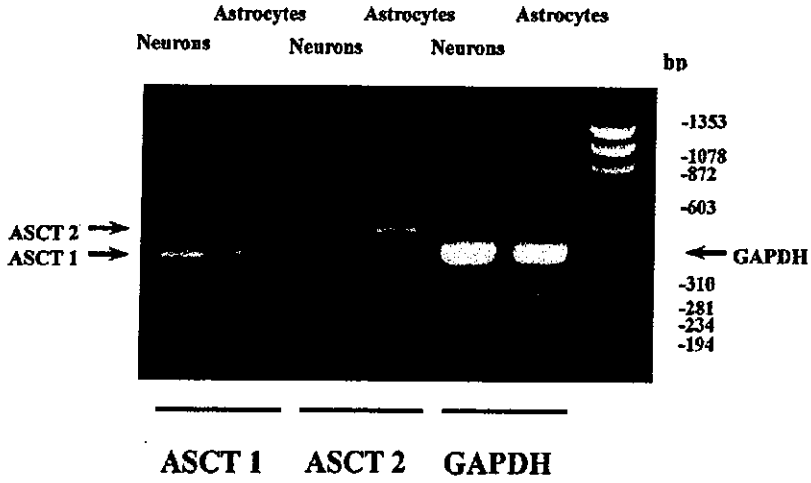

Fig. 3. Expression of ASCT1 and ASCT2 mRNA in rat neurons and astrocytes in primary culture as analyzed by RT-PCR. Total RNA was prepared from the neurons and astrocytes, and RT-PCR was performed as described in Fig. 2. GAPDH was used as a control. PCR products specific to ASCT1 (399 bp), ASCT2 (478 bp), and GAPDH (380 bp) were separated on a 3% agarose gels and stained with ethidium bromide. The right lane contains a molecular size marker.

inhibition experiments in which uptake of 20 nM L-[^{3}H] serine was measured in the presence of various 0.5 mM amino acids. The L-[^{3}H] serine uptake was strongly inhibited by neutral amino acids, i.e., L-alanine, L-serine, L-cysteine,

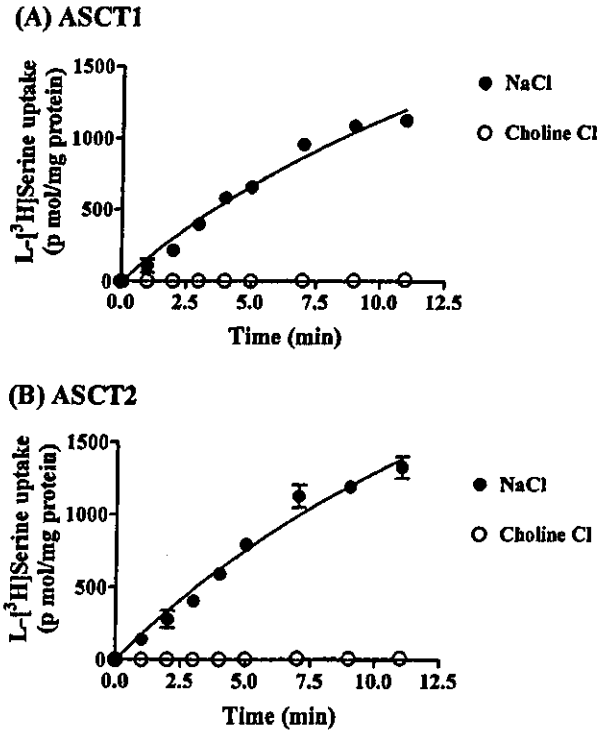

Fig. 4. Time course of L-[^{3}H] serine uptake into HEK293 cells transfected with ASCT1 (A) or ASCT2 (B). The uptake of L-[^{3}H] serine was measured at 37 °C for the indicated times in the presence of NaCl or choline chloride. Data are mean ± S.E.M. of four independent experiments done in quadruplicate.

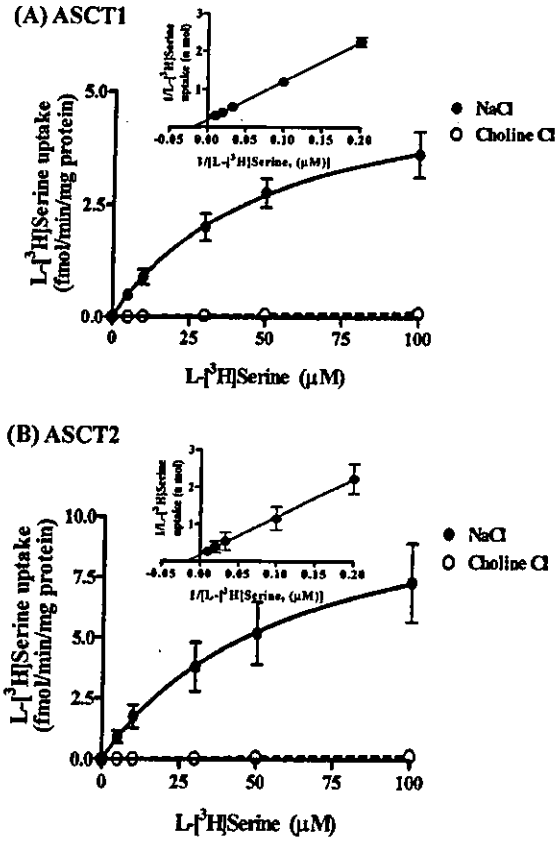

Fig. 5. Saturation analysis of Na^+-dependent L-[^{3}H] serine uptake into HEK293 cells transfected with rat ASCT1 (A) or ASCT2 (B) cDNA. Uptake was measured in the presence of increasing concentrations of L-[^{3}H] serine. Na^+-dependent (NaCl) and Na^+-independent (Choline chloride) uptake are presented as the mean±S.E.M. of quadruplicate determinations. The data represent a typical result out of four independent experiments. Nonspecific uptake was determined in the presence of 1 mM L-serine. Inset: Lineweaver-Burk plots used to calculate K_m and V_{max} values.

and L-threonine, in HEK293 cells transfected with either ASCT1 or ASCT2 (Fig. 6A and B). Consistent with the previous data on human ASCT1 (Arriza et al., 1993) and mouse ASCT2 (Utsunomiya-Tate et al., 1996), similar inhibitions were observed with L-asparagine, L-valine, and L-proline for rat ASCT1 and with L-asparagine, L-valine, L-methionine, glycine, L-glutamine, and L-leucine for rat ASCT2. Inhibitors for amino acid transporter system A and system L, α-(aminomethyl)isobutyric acid (MeAIB) and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), respectively, did not inhibit L-[^{3}H] serine uptake in HEK293 cells transfected with either ASCT1 or ASCT2 ($N = 1$).

In primary cultures of neurons or astrocytes, the uptake of L-[^{3}H] serine was also observed to occur by high-affinity (K_m = 15.0 ± 1.6 or 17.2 ± 2.1 μM; mean ± S.E.M, $N = 5$, respectively) transporters and was almost completely Na^+-dependent, which also indicated a single uptake site (Fig. 7). To evaluate the contribution of both ASCT1 and ASCT2 to the L-[^{3}H] serine uptake by neurons, we further examined the substrate specificity of the cultured neurons and astrocytes. Likewise, the neutral amino acids L-alanine, L-serine, L-cysteine, and L-threonine strongly inhibited the L-[^{3}H] serine uptake by both the neurons and astrocytes (Fig. 6C and D). Furthermore, MeAIB and BCH did not inhibit the uptake by either one. The inhibition experiments by using the neutral amino acids L-serine, L-alanine, L-cystein, or L-threonine as a competition amino acid on L-[^{3}H] serine uptake showed single site of uptake in HEK293 cells transfected with ASCT1 or ASCT2 ($N = 1$), cultured neurons, and astrocytes (Yamamoto et al., 2003). The inhibition profile for L-[^{3}H] serine uptake by neurons quite resembled that for HEK293 cells transfected with ASCT1, i.e., L-asparagine, L-valine, and L-proline, but not the other amino acids, also inhibited the L-[^{3}H] serine uptake in both rat primary neurons and HEK293 cells transfected with ASCT1. For astrocytes, the inhibition profile was different from that for primary neurons; for L-methionine, glycine, L-glutamine, and L-leucine were also weakly inhibitory, which partially resembled the situation for ASCT2. L-Proline also inhibited the L-[^{3}H] serine uptake by astrocytes, as it did that by ASCT1-transfected cells.

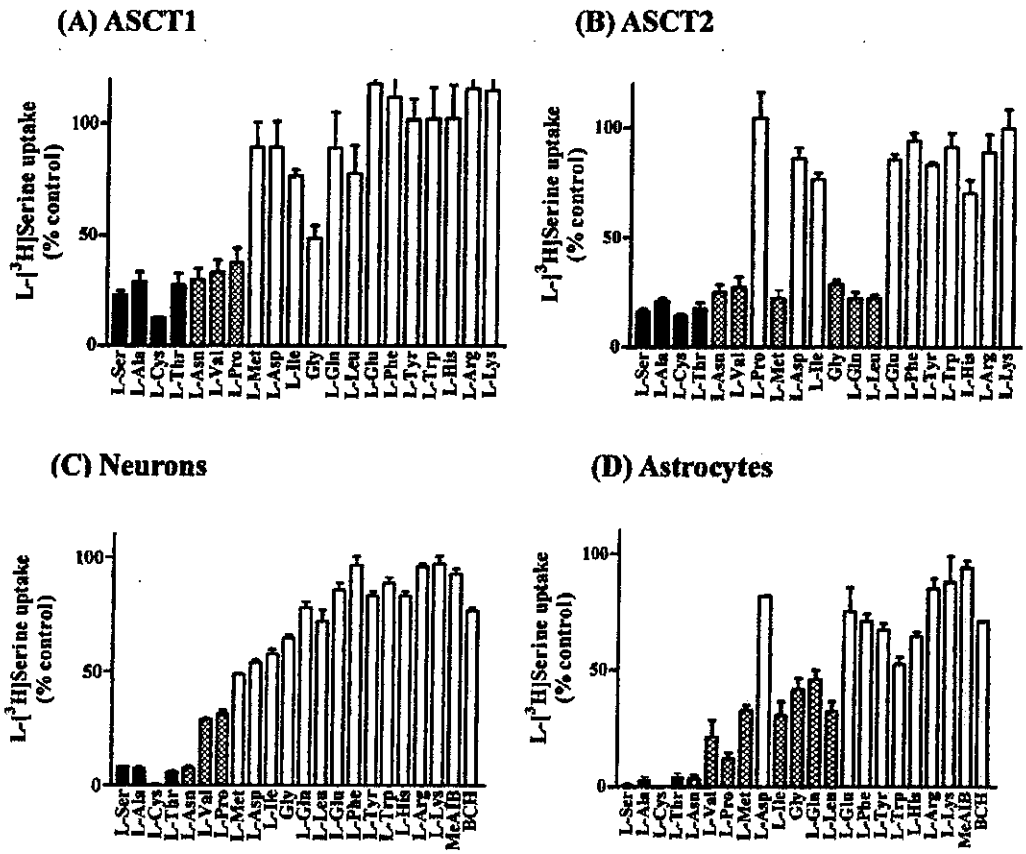

Fig. 6. Amino acid inhibition of L-[^{3}H] serine uptake by HEK293 cells transfected with rat ASCT1 (A) or ASCT2 (B), and by primary cultures of rat neurons (C) and astrocytes (D), respectively. The uptake of 20 nM L-[^{3}H] serine was measured for 5 min in Na^+-containing HEPES uptake buffer in the presence of the indicated amino acids (0.5 mM). The values represent the mean ± S.E.M. of 4–5 independent experiments, each done with quadruplicate determinations.

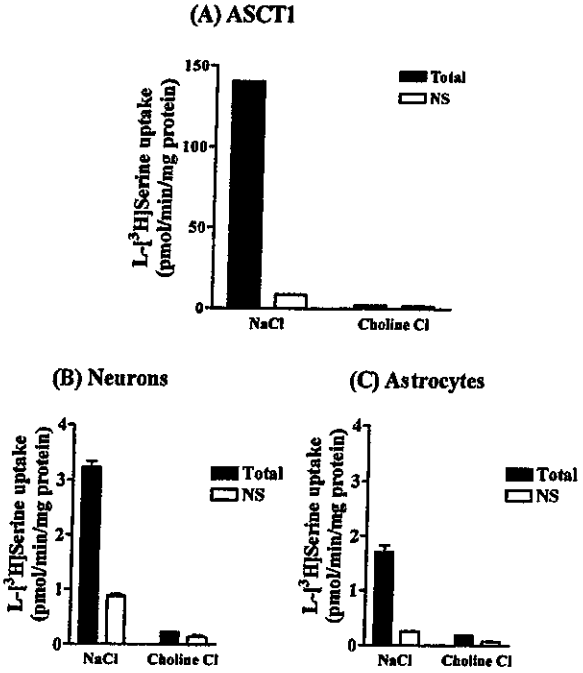

Fig. 7. Na^+ dependency of L-[^{3}H] serine uptake in HEK293 cells transfected with rat ASCT1 (A) and in primary cultures of rat neurons (B) and astrocytes (C). L-[^{3}H] serine uptake was measured in the presence of either NaCl or choline chloride, and the nonspecific uptake (NS) was measured in the presence of 1 mM L-serine. In each case the L-[^{3}H] serine uptakes was completely dependent on Na^+. Data represent the mean ± S.E.M. of four independent experiments, each done with quadruplicate determinations.

3.4. Northern blot analysis

Northern blot analysis was carried out to examine the regional variation in the expression of ASCT1 mRNA in the rat brain. Two hybridizing bands of 5.2 and 3.3 kb, and a weak band with the size corresponding to 1.6 kb were ubiquitously detected in various brain regions (Fig. 8). The 5.2 kb mRNA transcript appeared to be abundantly expressed in the striatum, hippocampus, and olfactory bulbs (Fig. 8C). In the cerebral cortex and cerebellum, this transcript were relatively less expressed than in other brain regions, but the 3.3 kb one was most abundantly expressed in them.

4. Discussion

In this communication, we have reported the cDNA cloning, expression, and functional characterization of rat ASCT1, and described the general distribution of the ASCT1 mRNA in the brain. The results presented here provide evidence that L-serine is predominantly taken up through the ASCT1 transporter in primary cultures of rat neurons. To clarify the role of ASCT1 in brain, we cloned and functionally expressed rat ASCT1 cDNA in HEK293 cells. The rat ASCT1 protein was 92 and 84% homologous to its mouse and human counterparts, and lacked three amino acids found at the N-terminal region of them. The most interesting difference among the rat, mouse, and human ASCT1 sequences is the loss of these three amino acids in rat ASCT1. However, there was no significant difference found in the affinity for L-serine (K_m = 88 μM for human (Arriza et al., 1993) and K_m = 67 μM for rat in the present study) or in the selectivity of amino acids between human and rat ASCT1s. At present, it is not clear whether the difference at the N-terminal region may affect the transport function or not. Thus, further detailed studies are needed to find out the significance of the role of the N-terminal regions of ASCT1.

To identify the transport systems for L-serine in neurons and astrocytes, we functionally expressed rat ASCT1 or rat ASCT2 in HEK293 cells, and compared their transport activity with that of rat neurons and of astrocytes by using L-[^{3}H] serine as a substrate. In agreement with previous reports on human ASCT1 (Arriza et al., 1993), rat ASCT1 exhibited Na^+ dependency and high affinity for neutral amino acids, i.e., L-alanine, L-cysteine, and L-threonine in addition to L-serine. The present study also showed that substitution of choline chloride for NaCl in the uptake buffer resulted in almost complete loss of L-[^{3}H] serine uptake by HEK293 cells transfected with rat ASCT1. Moreover, HEK293 cells that expressed rat ASCT2 also exhibited the Na^+ dependency and high affinity for neutral amino acids similar to rat ASCT1. In addition, the uptake of L-[^{3}H] serine by the rat neurons and astrocytes also occurred in a Na^+-dependent manner. Thus, participation of the Na^+-independent transporter systems, such as system L and system ASC, in the uptake of L-[^{3}H] serine by neurons in primary culture appears to be negligible. In addition to the system ASC, system A also is a candidate of Na^+-dependent uptake of L-serine in neurons and astrocytes; however, the lack of inhibition by a system A-specific substrate, MeAIB, indicates that the system A does not contribute to the uptake of L-serine by cultured neurons. In the primary cultures of rat neurons, L-asparagine, L-valine, and L-proline also inhibited L-[^{3}H] serine uptake, as did L-alanine, L-serine, L-cysteine, and L-threonine. These characteristics are consistent with those of ASCT1. The most striking difference in the substrate specificity for the uptake of L-[^{3}H] serine between neurons and astrocytes was that L-methionine, glycine, L-glutamine, L-leucine, and L-isoleucine weakly inhibited the uptake of L-[^{3}H] serine in astrocytes, which resembled the profile of ASCT2.

It has been ascertained that both ASCT1 and ASCT2 behave as electroneutral transporters which mediate a Na^+-dependent obligatory exchange of a substrate (Zerangue and Kavanaugh, 1996). High concentrations of L-serine can be observed in astrocytes (Verleysdonk and Hamprecht, 2000; Yasuda et al., 2001) and may serve as a counter substrate for exchange with extracellular presence of neutral amino acid ASCT substrates. In addition, L-serine fluxes are coupled to counter movement of L-glutamine or D-serine through

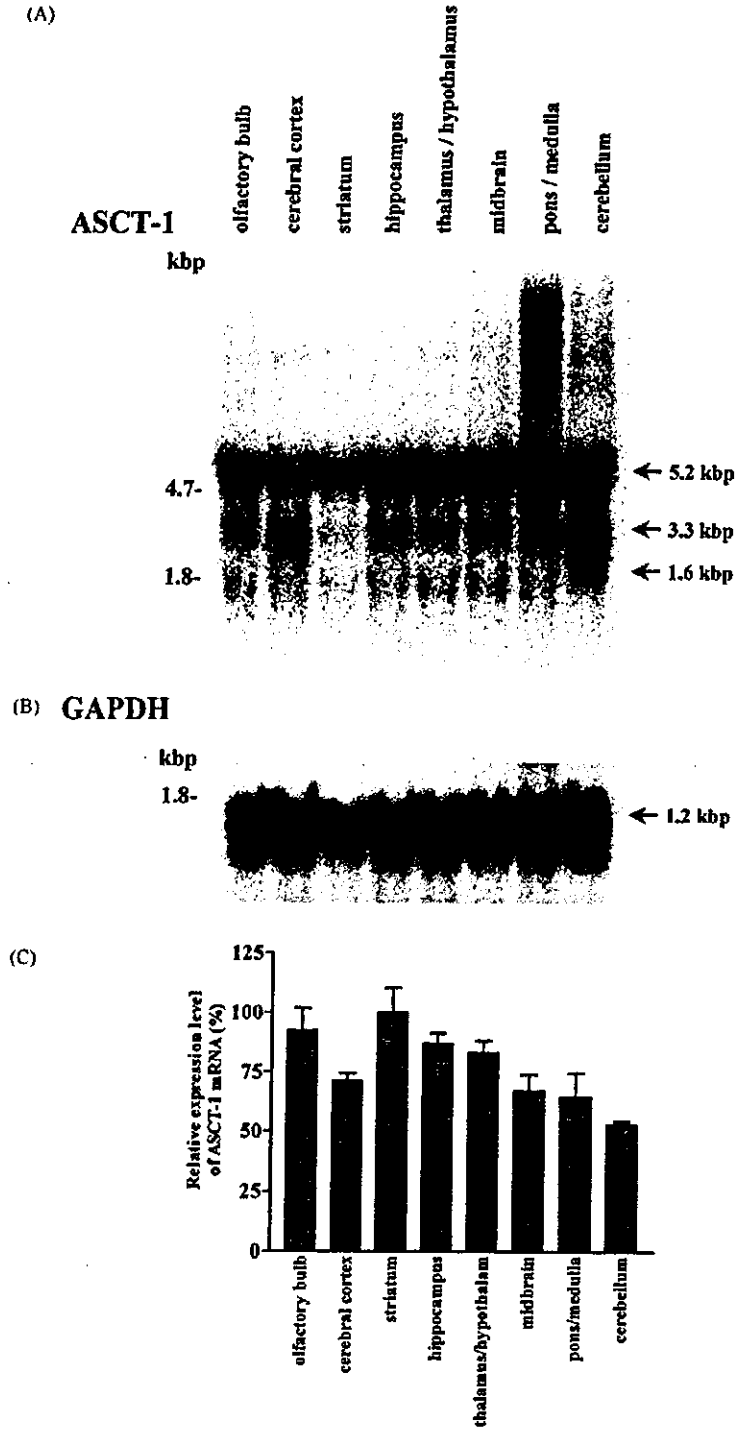

Fig. 8. Regional distribution of ASCT1 mRNA expression in rat brain. (A, B) High-stringency Northern blot analysis with total RNA (30 μg) from the indicated regions of rat brain was hybridized with a ^{32}P-labeled DNA probe for rat ASCT1 cDNA as described under Section 2. Hybridization with a GAPDH cDNA probe was used as a control. The positions of the molecular size are shown on the left. (C) The intensity of hybridized bands (5.2 kb mRNA) in "A" and "B" were densitometrically quantified. The expression level of ASCT1 mRNA was normalized to that of GAPDH mRNA in each region. The maximum expression in the striatum was set as 100%. The values are shown as the mean ± S.E.M. of three independent experiments.

the ASCT2 in cultured astrocytes (Broer et al., 1999) or C6 glioma cells (Hayashi et al., 1997), respectively. Thus, the accumulation of high concentrations of L-serine in the extracellular spaces resulted from the increase in the efflux of L-serine from astrocytes might facilitate the uptake of L-serine through ASCT1 in exchange with other ASCT substrates in neurons. In these manners, ASCT1 may participate in the regulation of intracellular concentration of L-serine in neurons and astrocytes.

ASCT1, but not ASCT2, was reported to be highly expressed in human and mouse brains (Arriza et al., 1993; Sakai et al., 2003). This high expression of ASCT1 in the brain suggests a particularly interesting role for L-serine there, as well as for another neutral amino acid, L-cysteine. Our analysis of mRNA expression of ASCT1 and ASCT2 in the cultured neurons and astrocytes by RT-PCR confirmed previous findings (Broer et al., 1999) and our recent results showing that ASCT2 was not expressed in rat neurons in primary culture (Yamamoto et al., 2003). Therefore, we conclude that the ASCT1 subtype only operates among the members of the system ASC transporter family in these cultured neurons.

ASCT1 mRNA was ubiquitously expressed in the brain. The relatively strong expression of ASCT1 in the striatum renders it a likely candidate as a modulator system of L-serine-mediated striatal functions. These findings are supported by membrane binding experiments using L-[^{3}H] serine as a ligand to detect L-serine transporter sites in membrane preparations, which revealed that the striatum showed a higher B_{max} value than other brain regions, such as the cerebral cortex, hippocampus, and cerebellum (T. Yamamoto, M. Shimizu, S. Furuya, Y. Hirabayashi, K. Takahashi, S. Okuyama, H. Yamamoto, unpublished observations). Furthermore, the intracerebroventricular administration of L-serine caused a higher increase in the L-serine level in the striatum than in the cerebral cortex or hippocampus, all of which increases peaked at 2 h (Hashimoto, 2002). It is likely that the expression of ASCT1 mRNA is correlated with the activity of L-serine uptake. Further studies on the role of ASCT1 in the striatum will be of interest.

In conclusion, we cloned rat ASCT cDNA and characterized the ASCT1. The present results indicate that ASCT1 seem to be the predominant player in the major mechanism of L-serine transport into rat neurons in primary culture.

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Regional Differences in Extracellular Dopamine and Serotonin Assessed by *In Vivo* Microdialysis in Mice Lacking Dopamine and/or Serotonin Transporters

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Cocaine conditioned place preference (CPP) is intact in dopamine transporter (DAT) knockout (KO) mice and enhanced in serotonin transporter (SERT) KO mice. However, cocaine CPP is eliminated in double-KO mice with no DAT and either no or one SERT gene copy. To help determine mechanisms underlying these effects, we now report examination of baselines and drug-induced changes of extracellular dopamine (DA_{ex}) and serotonin ($5\text{-}HT_{ex}$) levels in microdialysates from nucleus accumbens (NAc), caudate putamen (CPu), and prefrontal cortex (PFc) of wild-type, homozygous DAT- or SERT-KO and heterozygous or homozygous DAT/SERT double-KO mice, which are differentially rewarded by cocaine. Cocaine fails to increase DA_{ex} in NAc of DAT-KO mice. By contrast, systemic cocaine enhances DA_{ex} in both CPu and PFc of DAT-KO mice though local cocaine fails to affect DA_{ex} in CPu. Adding SERT to DAT deletion attenuates the cocaine-induced DA_{ex} increases found in CPu, but not those found in PFc. The selective SERT blocker fluoxetine increases DA_{ex} in CPu of DAT-KO mice, while cocaine and the selective DAT blocker GBR12909 increase $5\text{-}HT_{ex}$ in CPu of SERT-KO mice. These data provide evidence that (a) cocaine increases DA_{ex} in PFc independently of DAT and that (b), in the absence of SERT, CPu levels of $5\text{-}HT_{ex}$ can be increased by blocking DAT. Cocaine-induced alterations in CPu DA levels in DAT-, SERT-, and DAT/SERT double-KO mice appear to provide better correlations with cocaine CPP than cocaine-induced DA level alterations in NAc or PFc.
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Keywords: dopamine; serotonin; monoamine transporter; cocaine reward; knockout mice; *in vivo* microdialysis

INTRODUCTION

Cocaine increases extracellular levels of dopamine (DA), serotonin (5-HT) and norepinephrine (NE) by blocking the neural plasma membrane transporters for those neurotransmitters. Increased extracellular DA (DA_{ex}) levels in mesocorticolimbic DA systems have been postulated to mediate cocaine reward (Kuhar *et al*, 1991; Koob and Nestler, 1997; Bardo, 1998; Kelley and Berridge, 2002). However, homozygous dopamine transporter (DAT) knockout (KO) mice (DAT−/− mice) express intact cocaine reward in conditioned place preference (CPP) (Sora *et al*, 1998) and drug self-administration paradigms (Rocha *et al*, 1998). Cocaine reward is eliminated in double-KO mice with no DAT gene copies and either no or one copy of the SERT gene (Sora *et al*, 2001), but not in double-KO mice with neither DAT nor NET gene copies (Hall *et al*, 2002). Further, serotonin transporter (SERT) blockade with fluoxetine or norepinephrine transporter (NET) blockade with nisoxetine can yield rewarding effects in DAT-KO mice, which are never seen in wild-type animals (Hall *et al*, 2002).

We and others have postulated that the retention of cocaine reward in DAT-KO mice may be due to (a) roles for non-DA systems in normal cocaine reward and (b) adaptations to the lifelong loss of DAT found in DAT-KO mice (Kirkpatrick, 2001; Sora *et al*, 2001; Uhl *et al*, 2002). Some of these adaptive changes could come from involvement of redundant monoaminergic systems in cocaine

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reward. Since each transporter displays significant affinities for each monoamine (Faraj *et al*, 1994; Giros *et al*, 1994; Gu *et al*, 1994; Eshleman *et al*, 1999), the absence of its cognate transporter might allow a monoamine to diffuse further from its site of release and be accumulated by another transporter.

Cocaine and selective norepinephrine transporter (NET) blockers (eg reboxetine) are each reported to increase DA_{ex} in NAc of DAT-KO mice, suggesting that NET could act as an alternative uptake site for DA in such animals and that NET blockade might be a mechanism for both the cocaine- and nisoxetine-induced rewards found in DAT-KO mice (Carboni *et al*, 2001; Hall *et al*, 2002). However, *in vitro* data fail to identify cocaine influences on CPu or NAc DA uptake in DAT-KO mice (Budygin *et al*, 2002; Moron *et al*, 2002). The simple idea that NET mediates cocaine reward in the absence of DAT is also incompatible with observations that cocaine reward is ablated in DAT/SERT double-KO mice that express normal levels of NET (Sora *et al*, 2001).

Roles for 5-HT systems in cocaine reward (or aversion) are also less than clear from current data (Cunningham and Callahan, 1991; Kleven *et al*, 1995; Rocha *et al*, 1997; Kleven and Koek, 1998; Lee and Kornetsky, 1998; Parsons *et al*, 1998; Shippenberg *et al*, 2000; Baker *et al*, 2001; Sasaki-Adams and Kelley, 2001). Homozygous SERT-KO mice display enhanced cocaine CPP that is increased even more in combined SERT/NET double-KO mice (Sora *et al*, 1998; Hall *et al*, 2002). SERT-KO mice, in themselves and in combination with DAT-KOs, thus provide interesting models in which to investigate 5-HT, DA, and 5-HT/DA interactions important for psychostimulant reward.

In this present study, we have therefore examined baselines and drug-induced changes of DA_{ex} and $5\text{-}HT_{ex}$ in several brain regions implicated in psychostimulant effects, the NAc, CPu and prefrontal cortex (PFc) in DAT-KO, SERT-KO, and both heterozygous and homozygous DAT/SERT double-KO mice. We have studied the effects of both the nonselective blocker cocaine and the selective SERT and DAT blockers fluoxetine and GBR12909. These investigations provide insights into adaptive processes found in these mice and into 5-HT, DA, and 5-HT/DA interactions of the possible importance for cocaine reward.

MATERIALS AND METHODS

Animals

Mutant mice lacking DAT, SERT, and littermate wild-type mice were obtained from heterozygote crosses on 129/C57 mixed genetic backgrounds. DAT/SERT double-KO mice were obtained by intercrossing single KO lines as described previously (Sora *et al*, 2001). DNA extracted from tail biopsies was genotyped using PCR. Mice were group-housed (two to four per cage) with food and water *ad libitum* in a room maintained at $22\pm2°C$ and $65\pm5\%$ humidity under a 12 h light–dark cycle. Male and female mice from 10–24 weeks old of each genotype group (*n*equals;4–8) were used in each experiment equally. All animal experiments were performed in accordance with the Guidelines for the Care of Laboratory Animals of the Tokyo Institute of Psychiatry.

For the CPu cocaine study, all the nine DAT × SERT genotypes were examined (DAT+/+ SERT+/+, DAT+/+ SERT+/−, DAT+/+SERT−/−, DAT+/−SERT+/+, DAT+/−SERT+/−, DAT+/−SERT−/−, DAT−/− SERT+/+, DAT−/−SERT+/−, and DAT−/−SERT−/−). For NAc and PFc cocaine studies and for fluoxetine CPu and NAc studies, the four homozygous genotypes were examined (wildtype, DAT−/−SERT+/+, DAT+/+SERT−/−, DAT−/−SERT−/−). GBR12909 effects on CPu $5\text{-}HT_{ex}$ levels were examined in wild-type and DAT+/+SERT−/− mice.

Surgery

Mice were stereotaxically implanted with microdialysis probes under sodium pentobarbital anesthesia (50 mg/kg) in CPu (anterior +0.6 mm, lateral +1.8 mm ventral −4.0 mm from bregma), NAc (anterior +1.2 mm, lateral +1.0 mm ventral −5.0 mm from bregma) or PFc (anterior +2.0 mm, lateral +0.5 mm ventral −3.0 mm from bregma) according to the atlas of Franklin and Paxinos (1997). Probe tips were constructed with regenerated cellulose membranes that provided 50 kDa molecular weight cutoffs, outer diameters of 0.22 mm, and membrane lengths of either 1 mm (NAc) or 2 mm (CPu and PFc) (Eicom, Kyoto, Japan). Dialysis probe placements were verified histologically at the ends of each experiment (Figure 1), and experimental data were excluded if the membrane portions of the dialysis probes lay outside the central CPu, medial PFc or NAc core or shell regions, respectively.

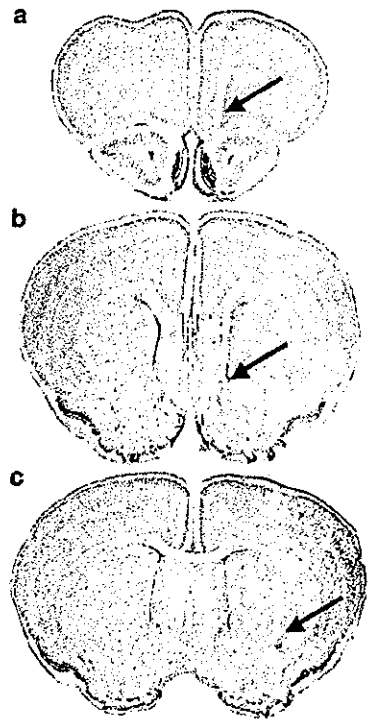

Figure 1 Location of dialysis probes in coronal sections of PFc (a), NAc (b), and CPu (c). The arrows illustrate the implantation sites of dialysis probes.

Microdialysis and Analytical Procedure

At 24 h after implantation, probes in freely moving mice were perfused with Ringer's solution (147 mM Na^+, 4 mM K^+, 1.26 mM Ca^{2+}, 1 mM Mg^{2+}, and 152.5 mM Cl^-, pH 6.5) at 1 µl/min for 180 min. DA_{ex} and 5-HT_{ex} baselines were obtained from average concentrations of three consecutive 10 min, 10 µl samples. These and subsequent 10 min, 10 µl dialysate fractions were analyzed using an AS-10 auto-injector (Eicom), high-performance liquid chromatography (HPLC), with a PPS-ODS reverse-phase column (Eicom) and a ECD-100 graphite electrode detector (Eicom). The mobile phase consisted of 0.1 M phosphate buffer (pH 5.5) containing sodium decanesulfonate (500 mg/l), EDTA (50 mg/l), and 1% methanol. Detection limits for DA and 5-HT were 1 fmol/sample with signal-to-noise ratios of at least 2. *In vitro* recoveries from the 1- and 2-mm membrane length probes were 10 and 15%, respectively.

Drugs

Test drugs were dissolved in saline for systemic administration or in Ringer's solution for local infusion via microdialysis probes. After establishment of stable baselines, cocaine HCl (10 mg/kg for subcutaneous injection or 100 µM for local infusion; Dainippon, Osaka, Japan), fluoxetine (20 mg/kg, Sigma, Tokyo, Japan), GBR12909 (10 mg/kg, Sigma) or saline (10 ml/kg) was administered subcutaneously (s.c.) and dialysates collected for 3 or 2 h, respectively.

Statistics

Baselines of DA_{ex} and 5-HT_{ex} were compared across genotype groups using two-way ANOVAs (DAT genotype, and SERT genotype). DA and 5-HT responses to drugs were expressed as percentages of baselines. Effects of drugs on DA_{ex} and 5-HT_{ex} were assessed by calculating the areas under time–response curves (AUC) for the first 120 or 180 min after drug administration. AUCs were analyzed using two-way ANOVAs (Drug, Genotype). Least significant difference tests were applied for multiple comparisons and *P*-values less than 0.05 were considered statistically significant. Statistical analyses used STATISTICA (StatSoft Inc., Tulsa, OK).

RESULTS

Baselines of DA_{ex} and 5-HT_{ex} in CPu, NAc, and PFc

The mean (±SEM) baselines of DA_{ex} and 5-HT_{ex} in dialysates from the CPu, NAc, and PFc in mice, who were subsequently treated with either vehicle or test drugs, are shown in Table 1. Two-way ANOVA of DA_{ex} baselines confirmed that DAT-KO had significant effects on DA_{ex} baselines in CPu ($F(1,91)=299.77$, $P<0.00001$) and NAc ($F(1,55)=101.49$, $P<0.00001$), but not PFc ($F(1,33)=0.07$, $P=0.79$). Dialysate DA in homozygous DAT-KO mouse CPu and NAc was approximately 10-fold higher than that in mice with either one or two copies of the DAT gene. 5-HT_{ex} baselines were unaffected by DAT-KO in any region.

SERT-KO exerted significant effects on 5-HT_{ex} baselines in each of these three regions ($F(2,91)=87.06$, $P<0.00001$; $F(1,55)=29.95$, $P<0.00001$; $F(1,33)=80.37$, $P<0.00001$, respectively). In CPu, NAc, and PFc, 5-HT_{ex} baselines in mice with no SERT gene were six to ten times as large as that found in mice with one or two copies of SERT gene. DA_{ex} baselines were unaffected by SERT-KO in any region.

Interestingly, there was a significant interaction between DAT and SERT genotype effects on basal NAc dialysate DA levels ($F(12,55)=4.33$, $P<0.05$). DA_{ex} levels in NAc of mice with no DAT or SERT genes (DAT−/−SERT−/−) were higher than those of mice with no DAT genes but two SERT genes (DAT−/−SERT+/+).

Systemic Cocaine Effects on DA_{ex} in CPu, NAc, and PFc

DA_{ex} level changes in CPu, NAc, and PFc following systemic cocaine administration are shown in Figure 2a, c, and e. DA responses to cocaine in the CPu of wild-type and DAT+/− mice peak at 40–60 min (Figure 2a). Cocaine also induces a slower DA response curve in the CPu of homozygous

Table 1 The Baselines (fmol/10 min) of DA_{ex} and 5-HT_{ex} in CPu, NAc and PFc

Genotype		CPu			NAc			PFc		
DAT	SERT	*n*	DA	5-HT	*n*	DA	5-HT	*n*	DA	5-HT
+/+	+/+	18	73.88±4.97	1.82±0.15	16	17.79±1.69	1.19±0.08	10	2.11±0.11	3.05±0.36
+/+	+/−	8	82.28±13.76	2.39±0.29						
+/+	−/−	13	69.82±7.12	16.33±2.58$^{*,+}$	15	16.23±2.23	15.50±4.20*	9	2.52±0.35	25.55±3.18*
+/−	+/+	9	79.69±12.32	1.28±0.13						
+/−	+/−	10	105.12±10.70	2.90±0.33						
+/−	−/−	9	93.04±14.09	17.73±3.30$^{*,+}$						
−/−	+/+	15	687.18±58.16$^{*,\#}$	1.61±0.10	14	188.17±23.00*	1.19±0.43	9	2.32±0.32	3.28±0.47
−/−	+/−	9	667.67±42.39$^{*,\#}$	2.38±0.31						
−/−	−/−	9	548.78±31.42$^{*,\#}$	12.78±1.46$^{*,+}$	14	275.34±38.47$^{*,\&}$	13.42±2.36*	9	2.22±0.15	17.06±2.61*

DA_{ex} or 5-HT_{ex} baselines were obtained from average concentrations (fmol/10 min) of three consecutive stable samples before injections. Values are the mean (±SEM) of baselines. $^*P<0.0001$ compared to wild-type mice; $^\#P<0.0001$ compared to mice with one copy of DAT gene; $^+P<0.0001$ compared to mice with one copy of SERT gene; $^\&P<0.001$ compared to DAT−/− mice.