ステムの異同は今後さらなる研究によって明らかになるであろうが，本総説ではオピオイドシステムとドーパミンシステムの差異に注目して論じた。オピオイドシステムはドーパミンシステムに影響することで快情動を発現するという仮説に対して否定的な実験結果が出されてきているが，オピオイドシステムによる快情動発現のメカニズムをうまく説明する他の仮説はまだ出されていない。精神的豊かさやQOL向上が注目される今日では，快情動発現メカニズムの解明は特に重要な研究課題であると考えられる。オピオイド研究は，受容体，神経ペプチド，鎮痛などの研究分野で従来から先導的な役割を果たしてきたが，今後も引き続き，心，情動の研究分野を牽引していく中心的な研究テーマであると考えられる。

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Effects of interferon-α on cloned opioid receptors expressed in *Xenopus* oocytes

Toru Kobayashi[a,*], Kazuo Washiyama[a], Kazutaka Ikeda[b]

[a]*Department of Molecular Neuropathology, Brain Research Institute, Niigata University, 1-757 Asahimachi, Niigata, Niigata 951-8585, Japan*

[b]*Department of Molecular Psychiatry, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan*

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Abstract

Interferon-α (IFNα) affects the opioid system. However, the direct action of IFNα on cloned opioid receptors remains unknown. Taking advantage of the functional coupling of cloned opioid receptors to G protein-activated inwardly rectifying K^+ (GIRK) channels in a *Xenopus* oocyte expression system, we investigated the effects of recombinant IFNα on cloned μ-, δ- and κ-opioid receptors. In oocytes co-injected with mRNAs for either the δ- or κ-opioid receptor and for GIRK channel subunits, IFNα at high concentrations induced small GIRK currents that were abolished by naloxone, an opioid-receptor antagonist, compared with the control responses to each selective opioid agonist. Additionally, IFNα induced no significant current response in oocytes injected with mRNA(s) for either opioid receptor alone or GIRK channels. In oocytes expressing the μ-opioid receptor and GIRK channels, IFNα had little or no effect. Moreover, in oocytes expressing each opioid receptor and GIRK channels, GIRK current responses to each selective opioid agonist were not affected by the presence of IFNα, indicating no significant antagonism of IFNα toward the opioid receptors. Furthermore, IFNα had little or no effect on the μ/δ-, δ/κ- or μ/κ-opioid receptors expressed together with GIRK channels in oocytes. Our results suggest that IFNα weakly activates the δ-and κ-opioid receptors. The direct activation of the δ- and κ-opioid receptors by IFNα may partly contribute to some of the IFNα effects under its high-dose medication.

Keywords: Interferon α; Opioid receptor; GIRK channel; *Xenopus* oocyte

* Corresponding author. Tel.: +81 25 227 0646; fax: +81 25 227 0818.
E-mail address: torukoba@bri.niigata-u.ac.jp (T. Kobayashi).

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Introduction

Interferons (IFNs) are multifunctional cytokines that possess antiviral, antitumor, antiproliferative and immunomodulatory actions mediated by specific cellular receptors (Maeyer and Maeyer-Guignard, 1998; Soos and Szente, 2003). Three major classes of IFNs are known; α, β and γ (Maeyer and Maeyer-Guignard, 1998). IFNα has been used for the treatment of chronic viral hepatitis, malignant tumors and several other diseases (Soos and Szente, 2003). However, treatment with IFNα also causes various side effects associated with the central nervous system (CNS), such as somnolence, confusion, depression, fever and sensory disturbance (Dafny, 1998). In animal studies, human IFNα was shown to bind to brain membranes (Janicki, 1992), and IFNα inhibited the binding of [^{3}H]naloxone, [^{3}H][D-Ala^2, D-Leu^5]enkephalin (DADLE) or [^{3}H]dihydromorphine to brain tissues (Blalock and Smith, 1981; Panchenko et al., 1987; Menzies et al., 1992). In addition, previous studies demonstrated immunological cross-reactivity between IFNα and γ-endorphin (Smith and Blalock, 1981), but not between it and β-endorphin (Epstein et al., 1982). Moreover, human IFNα, but not IFNβ or IFNγ, induced endorphin-like CNS effects, including analgesia and catalepsy (Blalock and Smith, 1981; Jiang et al., 2000). It also modulated immobility in a forced swimming test (Makino et al., 2000), wet-dog shakes induced by a $5HT_2$ receptor agonist (Kugaya et al., 1996), neuronal activity in several brain regions (Nakashima et al., 1987, 1988), electroencephalogram (EEG) activity (Birmanns et al., 1990; De Sarro et al., 1990) and hypothalamo-pituitary-adrenocortical secretory activity (Saphier et al., 1993, 1994). These effects were prevented or reversed by naloxone, an opioid receptor antagonist. Therefore, these observations suggest that the IFNα effects may be mediated by opioid receptors, which are implicated in many CNS functions as well as in immune and endocrine functions (Gutstein and Akil, 2001). However, the direct action of IFNα on cloned μ-, δ- and κ-opioid receptors remains unknown. In the present study, taking advantage of the functional coupling of cloned opioid receptors to G protein-activated inwardly rectifying K^+ (GIRK) channels in a *Xenopus* oocyte expression system (Ikeda et al., 1996; Ikeda et al., 2003), we investigated the effects of IFNα on the expressed opioid receptors.

Materials and methods

Preparation of specific mRNAs

Plasmids containing the entire coding sequences for the mouse μ-, δ and κ-opioid receptors and for the mouse GIRK1 and GIRK2 channel subunits were obtained previously (Ikeda et al., 1996; Kobayashi et al., 1995, 1999). The plasmids were linearized by digestion with an appropriate restriction enzyme. The specific mRNAs were synthesized in vitro from the linearized plasmids by using the mMESSAGE mMACHINE™ In Vitro Transcription Kit (Ambion, Austin, TX, USA).

Electrophysiological analyses

Adult female *Xenopus laevis* frogs were purchased from Copacetic (Soma, Aomori, Japan) and maintained in the laboratory until used. Frogs were anesthetized by immersion in water containing 0.15% tricaine (Sigma Chemical Co., St. Louis, MO, USA). A small incision was made on the abdomen to remove several ovarian lobes from the frogs that were humanely sacrificed after the final collection.

For *Xenopus* oocyte expression experiments (Kobayashi et al., 2002; Ikeda et al., 2003), *Xenopus laevis* oocytes (Stages V and VI) were isolated at 19 °C in Barth's solution (composition in mM: NaCl 88, KCl 1, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, $NaHCO_3$ 2.4, Tris-HCl 7.5 mM (pH 7.4), and 0.1 mg ml^{-1} gentamicin sulfate; Wako Pure Chemical Industries, Osaka, Japan), and injected with opioid-receptor mRNA(s) (~10 ng) and/or mRNAs for GIRK1 and GIRK2 subunits (each ~0.3 ng). The oocytes were defolliculated by manual dissection after treatment with 0.8 mg/ml collagenase (Wako Pure Chemical Industries, Osaka, Japan). Whole-cell currents were recorded from 2 to 5 days after the injection with a conventional two-electrode voltage clamp from oocytes which were superfused continuously with a high-potassium solution (composition in mM: KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1.5 and HEPES 5, pH 7.4 with KOH) at ~20 °C. The membrane potential was held at −70 mV. Microelectrodes were filled with 3 M KCl.

Drugs

The following opioid receptor ligands, purchased from Sigma Chemical Co. (St. Louis, MO, USA), were used: [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), a selective μ-opioid-receptor agonist; [D-Pen^{2,5}]enkephalin (DPDPE), a selective δ-opioid-receptor agonist; *trans*-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide (U50488H), a selective κ-opioid-receptor agonist; and naloxone, an opioid-receptor antagonist. Recombinant human IFNα-2b was purchased from Schering-Plough (Osaka, Japan). All drugs were dissolved in distilled water and added to the high-potassium solution in appropriate amounts before the experiment.

Statistical analysis of results

The values obtained are expressed as the mean ± S.E.M., and n is the number of oocytes tested. Statistical analysis of differences between groups was carried out by using paired t-test or Student's t-test. A probability of 0.05 was taken as the level of statistical significance.

Results

To investigate the effects of IFNα on the cloned μ-, δ- and κ-opioid receptors, we conducted *Xenopus* oocyte expression assays. In oocytes co-injected with either the δ- or κ-opioid receptor mRNA and GIRK1/GIRK2 mRNAs, application of 3000 IU/ml IFNα induced small inward currents, compared with the control responses to each selective opioid agonist (Fig. 1A). The current responses to IFNα were almost completely abolished by the simultaneous presence of 1 μM naloxone, an opioid-receptor antagonist, or fully abolished during application of 3 mM Ba^{2+} which blocks GIRK1/2 channels expressed in oocytes (Kobayashi et al., 2003) (n = 3 for each, data not shown). Furthermore, substitution of the perfusion solution containing additive distilled water used as the solvent vehicle, at the highest content (0.05%), caused no significant current response (n = 4; data not shown). In addition, in oocytes injected with either opioid receptor mRNA alone or GIRK1/GIRK2 mRNAs, neither IFNα nor selective opioid agonists induced any significant current response (n = 3, data not shown). These results suggest that IFNα weakly activated the δ- and κ-opioid receptors and that the current responses were mediated by GIRK channels. Furthermore, the magnitudes of the inward current responses induced by IFNα were

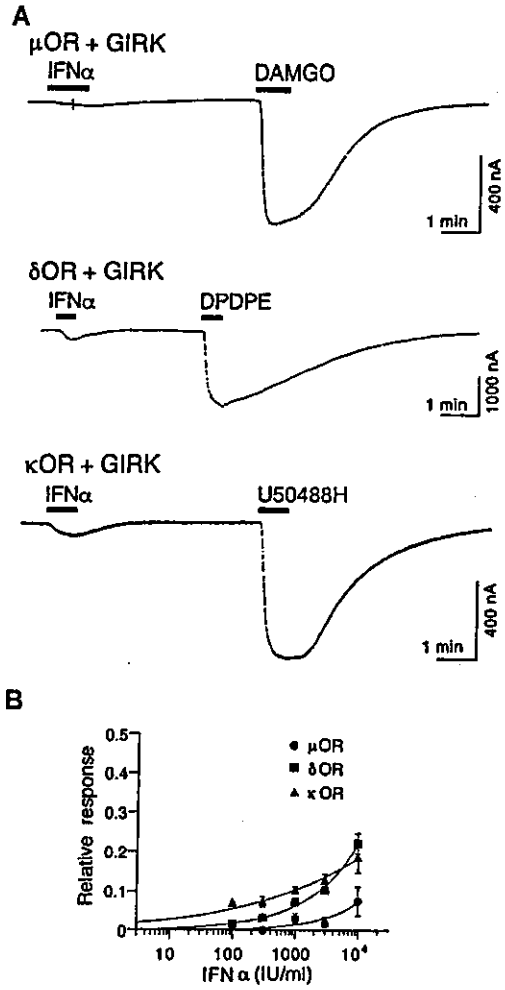

Fig. 1. Effects of IFNα on the cloned μ-, δ- and κ-opioid receptors. (A) Top: Current responses in an oocyte co-injected with the μ-opioid receptor (μOR) and GIRK1/GIRK2 mRNAs to 3000 IU/ml IFNα and to 300 nM DAMGO. Middle: Current responses in an oocyte co-injected with the δ-opioid receptor (δOR) and 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

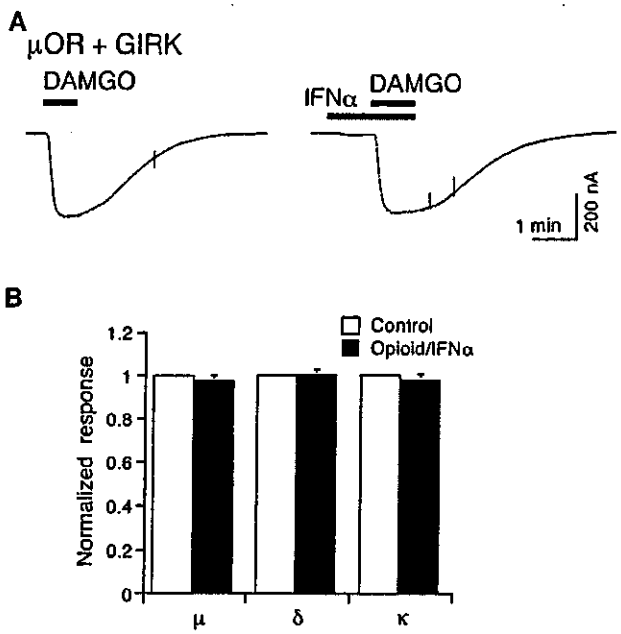

Fig. 2. Lack of antagonism of IFNα toward the cloned μ-, δ- and κ-opioid receptors. (A) Representative current responses in an oocyte co-injected with the μ-opioid receptor (μOR) and GIRK1/GIRK2 mRNAs to 200 nM DAMGO and to 200 nM DAMGO in the presence of 1000 IU/ml IFNα. Current responses were measured 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 ($n = 3$).

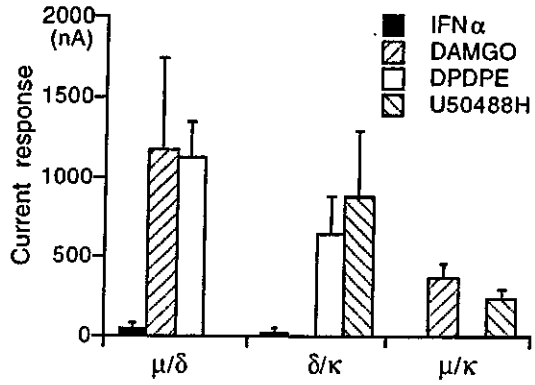

Fig. 3. Comparison of the effects of 3000 IU/ml IFNα and selective opioid receptor agonists: 300 nM DAMGO, 100 nM DPDPE and 100 nM U50488H, on oocytes expressing μ/δ-, δ/κ- or μ/κ-opioid receptors and GIRK1/2 channels ($n \geq 3$).

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.

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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☆

Toshifumi Yamamoto[a,*], Itone Nishizaki[a], Toshihide Nukada[b], Etsuko Kamegaya[b], Shigeki Furuya[d], Yoshio Hirabayashi[d], Kazutaka Ikeda[c], Harumi Hata[c], Hideaki Kobayashi[c], Ichiro Sora[c,e], Hideko Yamamoto[c]

[a] *Laboratory of Molecular Recognition, Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan*
[b] *Department of Neural Signaling, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-0057, Japan*
[c] *Department of Molecular Psychiatry, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-0057, Japan*
[d] *Neuronal Circuit Mechanism Research Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*
[e] *Division of Psychobiology, Department of Neuroscience, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Sendai 980-8574, Japan*

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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.

* Corresponding author. Tel.: +81-45-787-2336; fax: +81-45-787-2316.
E-mail address: yamamoto@yokohama-cu.ac.jp (T. Yamamoto).

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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/EcoRI* fragment from pASCT-2P were ligated with the 2.9 kb *SacI/EcoRI* fragment from pBluescript SK(+) (Stratagene) to yield pBlASCT-4P. The 880 bp *SacI/HindIII* fragment from pBlASCT-4P was ligated with the 4.2 kb *SacI/HindIII* 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 *KpnI* and *EcoRV* digestions and inserted into the *KpnI/EcoRV* sites of pcDNA3.1(+) to yield pCDASCT2.

2.4. Expression in HEK293 cells

The 1.6 kb blunted *EcoRI* fragment containing the entire coding region of the rat ASCT1 or ASCT2 was inserted into the *EcoRV* 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
ACGCCCGAGACGGGGACGAGCAAAGCGCAGCGCTGTGCTAGCTTTTTTCCGGCGCAACGCGCTGGTGCTGCTCACC   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
GTGTCGGGGGTGTTGGCGGGCGCCGGCATGGGCGCGGCGCTTCGCGGGCTGCAGCTCACCCGCACGCAAATCACT   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
TTAGGACTGGTCCTTTTGCCCTGATGTTAGGAGTGGCTCTAAAGAAGCTAGGCCCCGAGGGAGACGAGCTCATC   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
AACCCGTTCACGTTCCTCCTGGGCCTCCTCACCCCGTTTGCGACGGCTTTGCGACCTGTTCCAGCTCAGCAACC   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
CTCAACCACCTGAATCAGAGGACAGTGAAGAAGGGTGAGCAGGAGCTGCAAGAAGTGAAGGTGGAAGCCATTCCC   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).

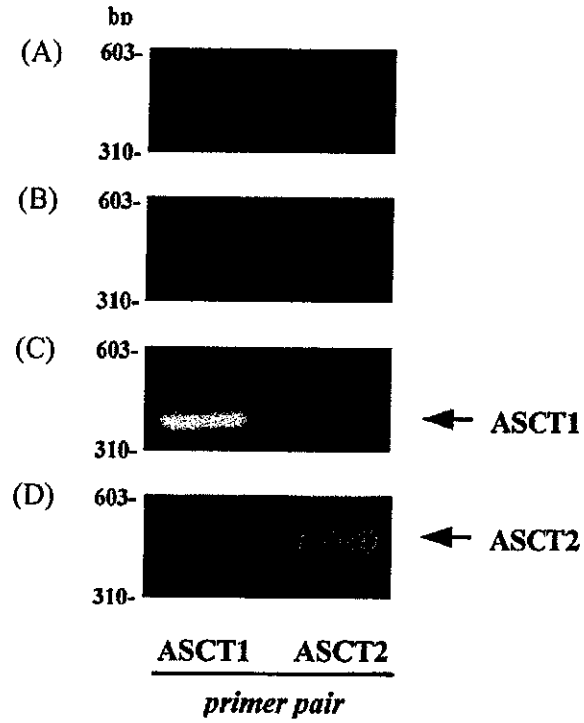

Fig. 2. Expression of rat ASCT1 and ASCT2 in HEK293 cells. Total RNA was prepared from untreated HEK293 cells (A) or those transfected with empty vector (B), ASCT1- (C), or ASCT2 cDNA (D). RT-PCR was performed to detect the expression of rat ASCT1 (left lane) or ASCT2 (right lane) by using specific primer 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

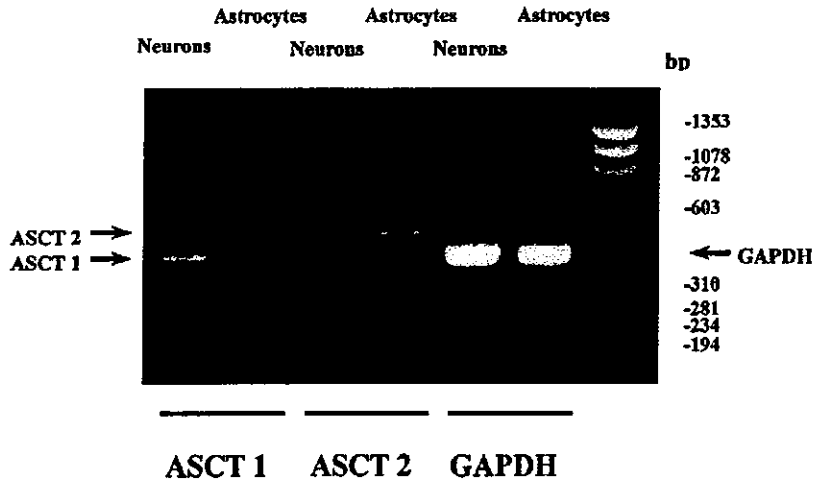

Fig. 3. Expression of ASCT1 and ASCT2 mRNA in rat neurons and astrocytes in primary culture as analyzed by RT-PCR. Total RNA was prepared from the neurons and astrocytes, and RT-PCR was performed as described in Fig. 2. GAPDH was used as a control. PCR products specific to ASCT1 (399 bp), ASCT2 (478 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,

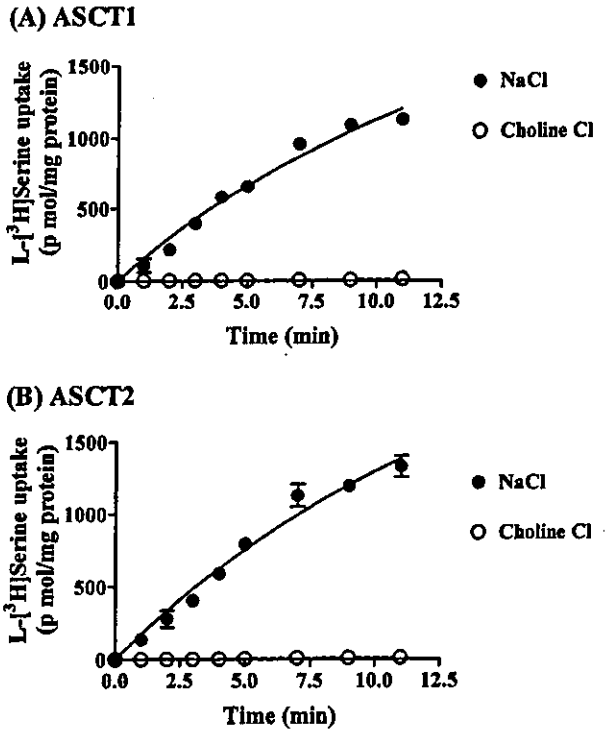

Fig. 4. Time course of L-[^{3}H] serine uptake into HEK293 cells transfected with ASCT1 (A) or ASCT2 (B). The uptake of L-[^{3}H] serine was measured at 37 °C for the indicated times in the presence of NaCl or choline chloride. Data are mean ± S.E.M. of four independent experiments done in quadruplicate.

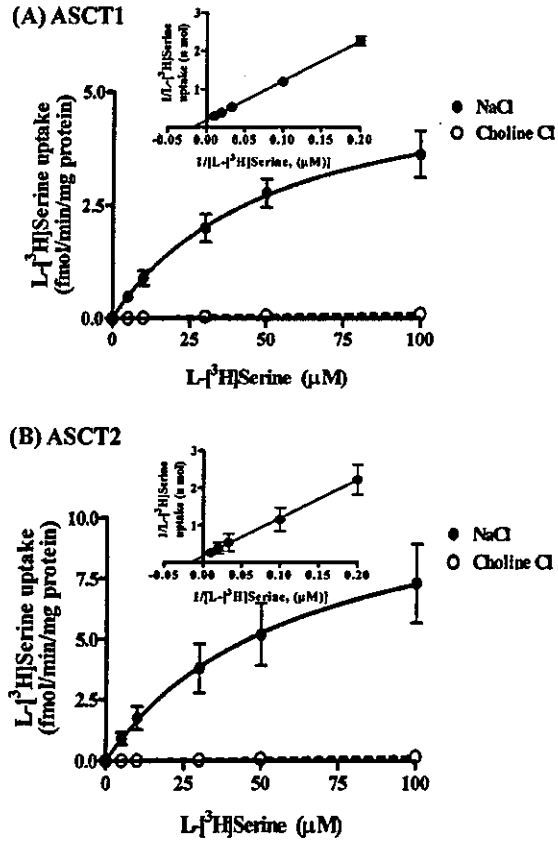

Fig. 5. Saturation analysis of Na^+-dependent L-[^{3}H] serine uptake into HEK293 cells transfected with rat ASCT1 (A) or ASCT2 (B) cDNA. Uptake was measured in the presence of increasing concentrations of L-[^{3}H] serine. Na^+-dependent (NaCl) and Na^+-independent (Choline chloride) uptake are presented 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.

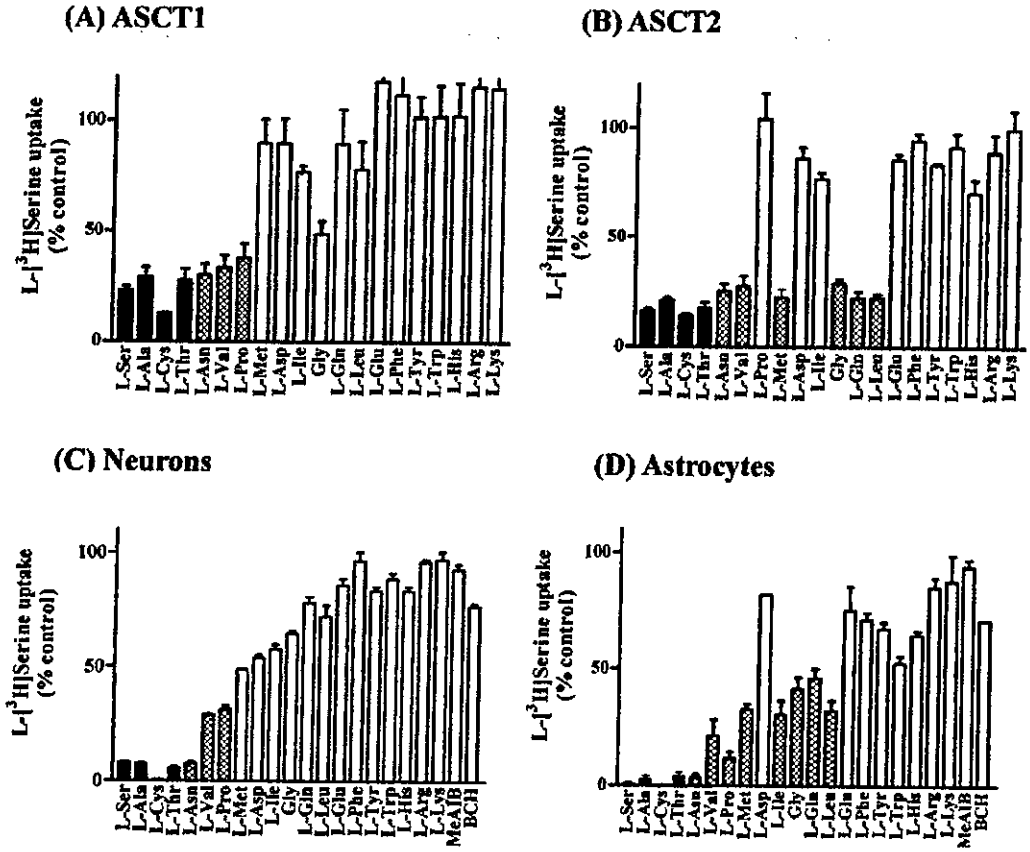

Fig. 6. Amino acid inhibition of L-[^{3}H] serine uptake by HEK293 cells transfected with rat ASCT1 (A) or ASCT2 (B), and by primary cultures of rat neurons (C) and astrocytes (D), respectively. The uptake of 20 nM L-[^{3}H] serine was measured for 5 min in Na^+-containing HEPES uptake buffer in the 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.

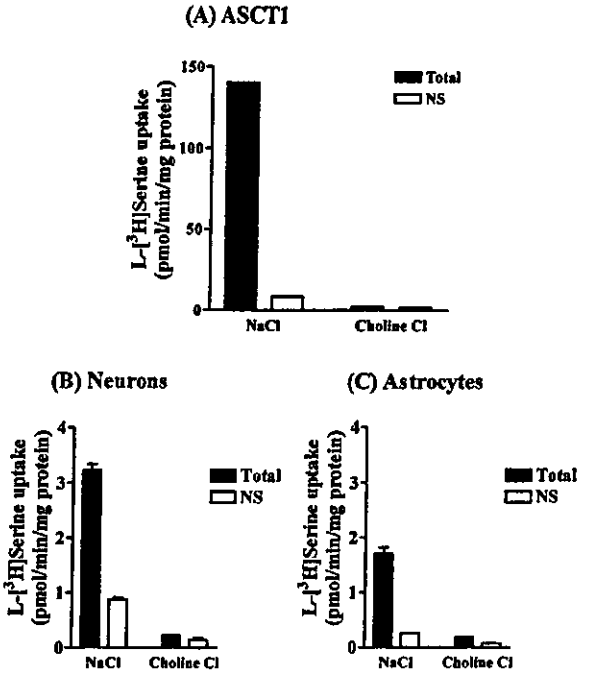

Fig. 7. Na^+ dependency of L-[^{3}H] serine uptake in HEK293 cells transfected with rat ASCT1 (A) and in primary cultures of rat neurons (B) and astrocytes (C). L-[^{3}H] serine uptake was measured in the presence of either NaCl or choline chloride, and the nonspecific uptake (NS) was measured in the 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

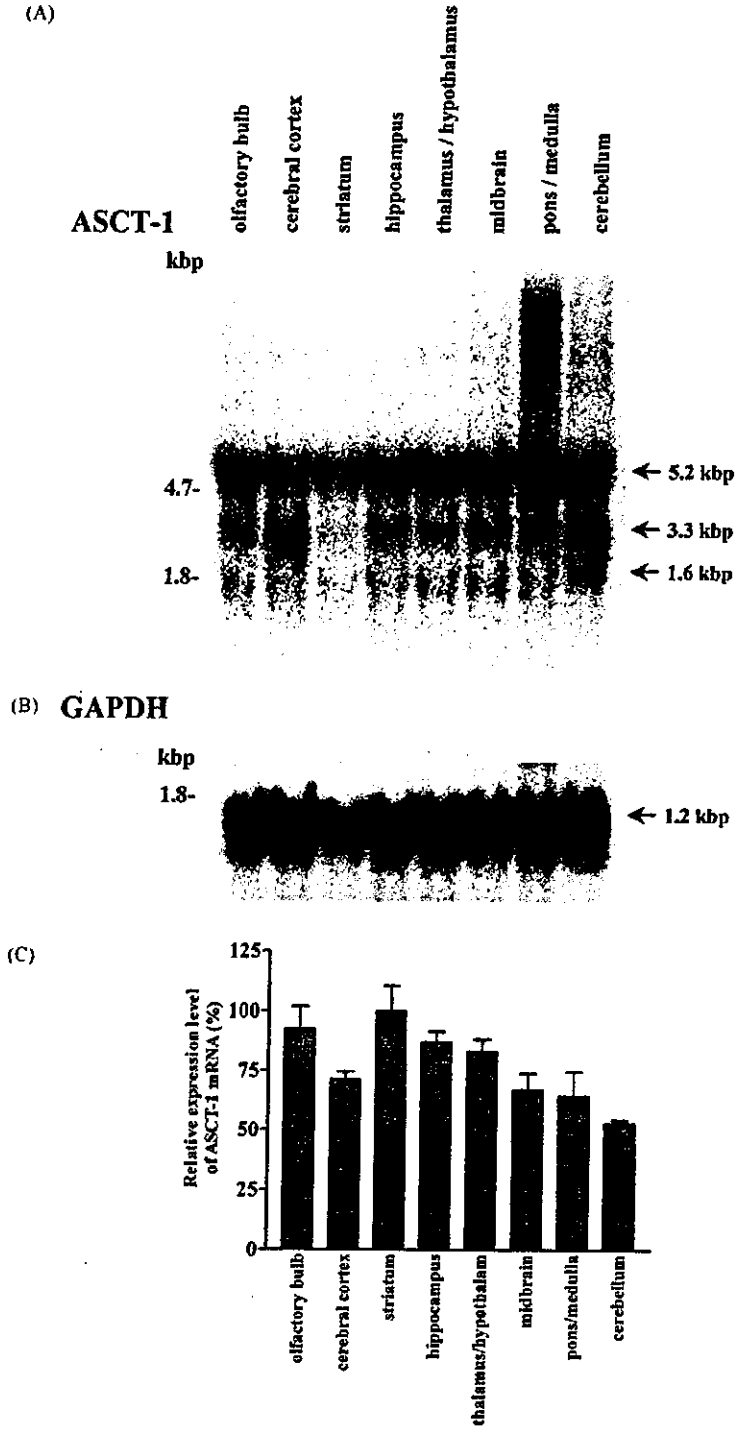

Fig. 8. Regional distribution of ASCT1 mRNA expression in rat brain. (A, B) High-stringency Northern blot analysis with total RNA (30 μg) from the indicated regions of rat brain was hybridized with a ^{32}P-labeled DNA probe for rat ASCT1 cDNA as described under Section 2. Hybridization with a GAPDH 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.