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Regulation of serotonin transporter gene expression in human glial cells by growth factors

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Abstract

The aims of this study were to identify monoamine transporters expressed in human glial cells, and to examine the regulation of their expression by stress-related growth factors. The expression of serotonin transporter mRNA was detected by reverse transcriptase-polymerase chain reaction in normal human astrocytes, whereas the dopamine transporter (DAT) and the norepinephrine transporter (NET) were not detected. The cDNA sequence of the "glial" serotonin transporter in astrocytes was consistent with that reported for the "neuronal" serotonin transporter (SERT). Moreover, we also demonstrated SERT expression in glial fibrillary acidic protein-positive cells by immunocytochemical staining in normal human astrocytes. Serotonin transporter gene expression was also detected in glioma-derived cell lines (A172, KG-1-C and KGK). Addition of basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) for 2 days increased serotonin transporter gene expression in astrocytes and JAR (human choriocarcinoma cell line). Basic fibroblast growth factor, but not epidermal growth factor, increased specific [^{3}H]serotonin uptake in astrocytes in a time (1–4 days)- and concentration (20–100 ng/ml)-dependent manner. The expression of genes for basic fibroblast growth factor and epidermal growth factor receptors was detected in astrocytes. These findings suggest that the expression of the serotonin transporter in human glial cells is positively regulated by basic fibroblast growth factor. © 2001 Published by Elsevier Science B.V.

Keywords: 5-HT (5-hydroxytryptamine, serotonin) transporter; 5-HT (5-hydroxytryptamine, serotonin) uptake; Glia; Basic fibroblast growth factor; Epidermal growth factor (human)

1. Introduction

In the central nervous system, to terminate neurotransmission, monoamines released from the nerve terminal are rapidly taken up by sodium- and chloride-dependent monoamine transporters, which are also located in the nerve terminal. Norepinephrine, dopamine and serotonin transporters (NET, DAT and SERT), which were recently cloned from neuronal cells, have 12 transmembrane domains and belong to the same superfamily (Pacholczyk et al., 1991; Shimada et al., 1991; Hoffman et al., 1991; Ramamoorthy et al., 1993).

Glial cells also take up monoamines in both sodium-dependent and sodium-independent manner (Streich et al., 1996; Inazu et al., 1999a). Recently, extraneuronal monoamine transporters (EMT or OCT3) were cloned as candidates of a glial monoamine transporter, and shown to take up dopamine or a neurotoxin, MPP^+, but not serotonin (Wu et al., 1998; Gründemann et al., 1998). Serotonin is also taken up into astrocytes (Katz and Kimelberg, 1985; Dave and Kimelberg, 1994), and SERT is expressed in cultured rat astrocytes (Bel et al., 1997; Hirst et al., 1998). However, the molecular characteristics and physiological functions of these glial transporters have not been elucidated.

It is well known that glial cells protect neurons against various stresses, such as ischemia, trauma or neurotoxins in the brain (Louw et al., 1998; Eggert et al., 1999; Raghavendra Rao et al., 2000). Under these conditions, it may be difficult for a neuron to take up excessively

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released monoamines due to neuronal dysfunction, leading to further neuronal damage as a result of the excitotoxicity of monoamines such as serotonin (Globus et al., 1992; Nakata et al., 1997; Joseph et al., 1992). However, it is possible that stress-resistant glial cells scavenge monoamines through glial monoamine transporters and suppress their neurotoxicity. In regions of brain damage, several growth factors such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) are known to be synthesized and released from activated glial cells (Eclancher et al., 1996; Hatten et al., 1991), and are reported to be involved in neuroprotective effects (Hicks et al., 1998).

Therefore, in the present study, we elucidated the properties of monoamine transporters, especially SERT, expressed in human cultured glial cells, and the possible regulation of SERT expression by bFGF and EGF. We discuss the physiological function of glial monoamine transporters.

2. Materials and methods

2.1. Cells

Normal human astrocytes were purchased from Clonetics (Walkersville, MD, USA) and were cultured in astrocyte basal medium (Clonetics) with 10% fetal bovine serum. KG-1-C (human mixed glioma) and A172 (human glioblastoma) cells were purchased from Health Science Research Resources Bank (Osaka, Japan). These cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY). KGK (human glioma) cells were provided by Prof. Tatsukawa (Showa University, Japan). JAR was purchased from ATCC (Rockville, MD, USA). KGK and JAR were cultured in RPMI1640 medium with 10% fetal bovine serum.

2.2. Reagents

[^{3}H]Serotonin (13.8 Ci/mmol) was purchased from NEN (Boston, USA). [^{32}P]dCTP (3000 Ci/mmol) was purchased from the Institute of Isotopes (Budapest, Hungary). Sertraline and citalopram were kindly donated by Pfizer (New York, USA) and Zeria Pharmaceutical (Tokyo, Japan), respectively. The following drugs were obtained from commercial sources: bFGF, EGF, imipramine and desipramine from Sigma (St. Louis, MO, USA); Isogen, gene *Taq* DNA polymerase and Seakem agarose from Nippon Gene (Tokyo, Japan); GeneAmp RNA Core kit from Perkin Elmer (Warrington, UK); ExpressHyb from Clontech (Palo Alto, CA, USA); and Megaprime DNA Labeling System and Hybribond-N from Amersham (Arlington Heights, IL, USA). The polyclonal antibody to SERT was obtained from DiaSorin (Minneapolis, USA) and the monoclonal antibody to glial fibrillary acidic protein (GFAP) was from Sigma. All other reagents were of analytical grade or the highest grade commercially available.

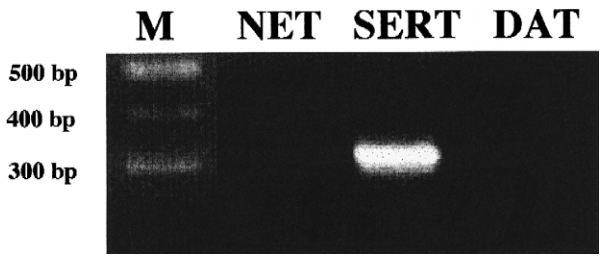

Fig. 1. Expression of serotonin transporter (SERT) mRNA in normal human astrocytes. Extracted total RNA from normal human astrocytes was amplified by RT-PCR using the specific primer shown in Table 1. PCR products were separated by electrophoresis on 3% agarose gels and visualized using ethidium bromide staining. M—molecular weight marker (300, 400 and 500 bp).

2.3. Detection of mRNA expression of monoamine transporters and FGF and EGF receptors

We examined whether mRNA for SERT, DAT, NET, bFGF receptors (FGFR1 and FGFR2) and the EGF receptor is expressed in normal human astrocytes and human glioma cell lines using the reverse transcriptase-polymerase chain reaction (RT-PCR) method.

Table 1
Specific oligonucleotide primers

	5′ Primer	3′ Primer	Length (bp)
NET	GCTTCTACTACAACGTCATCATC	CGATGACGACGACCATCAG	294
SERT	CATCTGGAAAGGCGTCAAG	CGAAACGAAGCTCGTCATG	319
DAT	TCATCTCACTGTATGTCGGC	CAGCACGATGACCAGCACC	311
EGFR	GCTACGATTGGCTGAAGTAC	ATTGGGTGTAGAGAGACTGGA	518
FGFR1	CATCATCTATTGCACAGGGG	AGTCTTTCTCTGTTGCGTCCG	442
FGFR2	CTCAACCAGAAGTGTACGTG	GCTCCTGCTTAAACTCCTTC	470
GAPDH	TGAAGGTCGGTGTCAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC	983

2.3.1. Total RNA extraction

The cells were cultured to confluence in a 10-cm dish. The culture medium was aspirated, and the cells were rinsed once with a phosphate buffer solution. One milliliter of Isogen was added to the dish, and total RNA was isolated according to the manufacturer's instructions. The amount of RNA was determined from the optical density measured at 260 nm.

2.3.2. RT-PCR reaction

cDNA was synthesized from 1.0 μg of total RNA with reverse transcriptase and random hexamer supplied in a GeneAmp RNA Core kit. PCR primers for human monoamine transporters (SERT, NET and DAT) were as previously reported (Lode et al., 1995). Primers specific for cloned EGFR, FGFR1 and FGFR2 were designed with a computer program (GENETYX-MAC ver 8.0, Software Development, Tokyo, Japan) and synthesized by GIBCO BRL. The sequences of the oligonucleotide primers used are shown in Table 1.

PCR was performed in a final volume of 25 μl, containing 2.5 mM dNTP mixture, 0.8-μM sense and antisense primers, 1 U gene *Taq* DNA polymerase and 1 μl cDNA in Gene *Taq* Universal Buffer (1.5 mM $MgCl_2$). The samples were placed in a Thermal Cycler MP (TAKARA, Kyoto, Japan), denatured at 94°C for 2 min, and amplified for 30, 34, and 40 cycles, for SERT, NET and DAT, respectively (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 90 s, and final elongation for 7 min at 72°C). For EGFR, FGFR1 and FGFR2, cDNA from normal human astrocytes was denatured at 94°C for 4 min and amplified for 40 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final elongation for 7 min at 72°C). The PCR products were separated by electrophoresis on 3% Seakem agarose gels and visualized using ethidium bromide staining.

The full cDNA sequence of a detected monoamine transporter was determined by sequencing, according to the method of Sanger et al. (1977) after 3′ and 5′ rapid amplification of cDNA end (RACE) procedures.

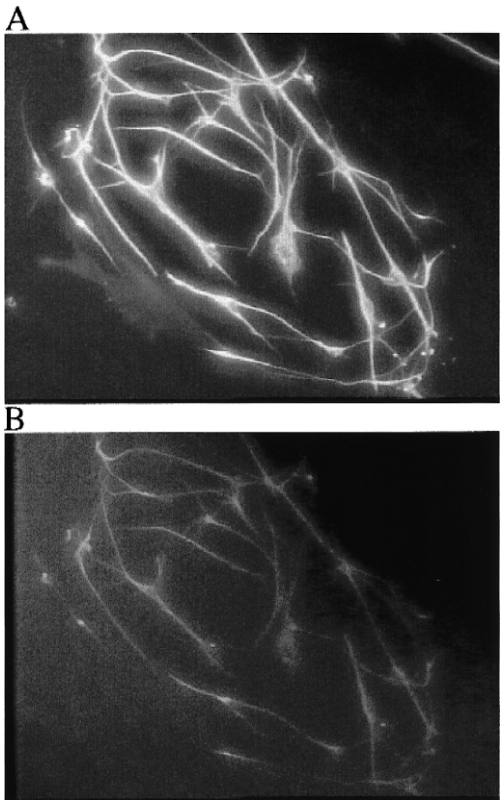

Fig. 3. Fluorescence micrographs showing double-immunolabeling of cultured normal human astrocytes. (A) GFAP immunoreactivity, (B) SERT immunoreactivity. For all, ×200.

2.4. Immunocytochemistry for serotonin transporter in normal human astrocytes

Normal human astrocytes were cultured to confluence in a six-well plate. The culture medium was aspirated, and the cells were rinsed twice with phosphate-buffered saline (PBS). One milliliter of −20°C methanol was added to a well for fixation, and then the cells were rinsed with PBS. For double immunostaining, the cells were preincubated with 10% normal goat serum and 0.3% Triton X-100 for 1 h, and then incubated with anti-SERT antibody and anti-

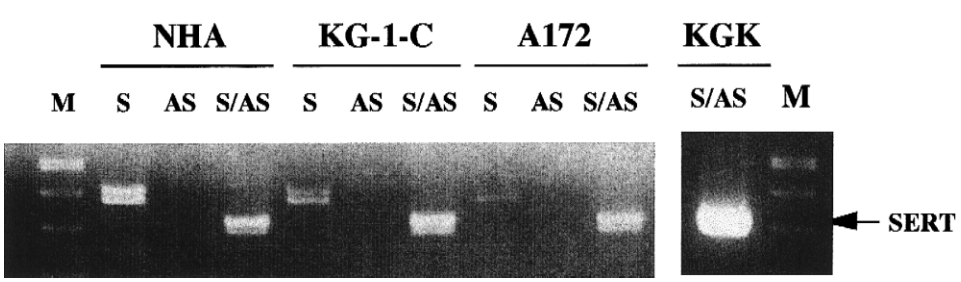

Fig. 2. Expression of SERT in human glioma cell lines. Extracted total RNA from normal human astrocytes (NHA) and human glioma cell lines was amplified by RT-PCR using the specific primer shown in Table 1. PCR products were separated by electrophoresis on 3% agarose gels and visualized using ethidium bromide staining. S—with 5′ (sense) primer, AS—with 3′ (antisense) primer, S/AS—with both 3′ and 5′ primers. M—molecular weight marker (300, 400 and 500 bp).

GFAP antibody for 72 h at 4°C. After being rinsed, the cells were incubated with Alexa 546-labeled goat anti-rabbit IgG antibody (X400, Molecular Probed, Eugene, OR, USA) for SERT and Alexa 488-labeled goat anti-mouse IgG antibody (X400, Molecular Probed) for GFAP at room temperature for 1 h. The cells were then examined with an Olympus AX-70 fluorescence microscope (Olympus, Tokyo, Japan).

2.5. Effects of bFGF or EGF on SERT expression and [^{3}H]serotonin uptake

bFGF or EGF (final 10, 20, 50 and 100 ng/ml) was added to the culture medium of normal human astrocytes, A172 and JAR, for 1–4 days. The treated cells were used for assays on SERT mRNA expression (RT-PCR and Northern blotting) and [^{3}H]serotonin uptake.

2.5.1. Northern blotting

Twenty micrograms of the total RNA from a 10-cm dish was applied to a single lane in 1.4% Seakem agarose gel. After electrophoresis, samples were transferred to hybridization transfer membranes (Hybribond-N) with 2 × saline–sodium citrate (SSC) buffer. The membranes were hybridized in Rapid-hyb buffer (ExpressHyb) at 68°C for 2 h with a [^{32}P]-labeled human SERT cDNA probe, which was prepared from [^{32}P]dCTP by using the Megaprime DNA labeling system. The membranes were washed twice at room temperature with 2 × SSC/0.05% sodium dodecyl sulfate (SDS) for 15 min, and twice at 42°C with 0.5 × SSC/0.1% SDS for 15 min. mRNA on the membrane was visualized using BAS3000 (FUJI, Japan).

2.5.2. [^{3}H] serotonin uptake assay

For the [^{3}H]serotonin uptake assay, normal human astrocytes and JAR were seeded in a poly-D-lysine coated six-well plate (Becton Dickinson, Franklin Lakes, NJ, USA) under sub-confluent conditions. The culture medium was removed and replaced by uptake buffer (122 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 0.4 mM $MgSO_4$, 20 mM HEPES, 1 mM ascorbic acid, 10 mM $NaHCO_3$, 0.1 mM pargyline, pH 7.4). Cells were preincubated in uptake buffer, with or without a reuptake inhibitor (imipramine or sertraline, final 10^{-10}–10^{-5} M), for 20 min at 37°C in humidified 5% CO_2 and 95% air. Subsequently, [^{3}H]serotonin (final 50 nM) was added, and the incubation was carried out for a further 20 min at 37°C. The uptake of [^{3}H]serotonin was

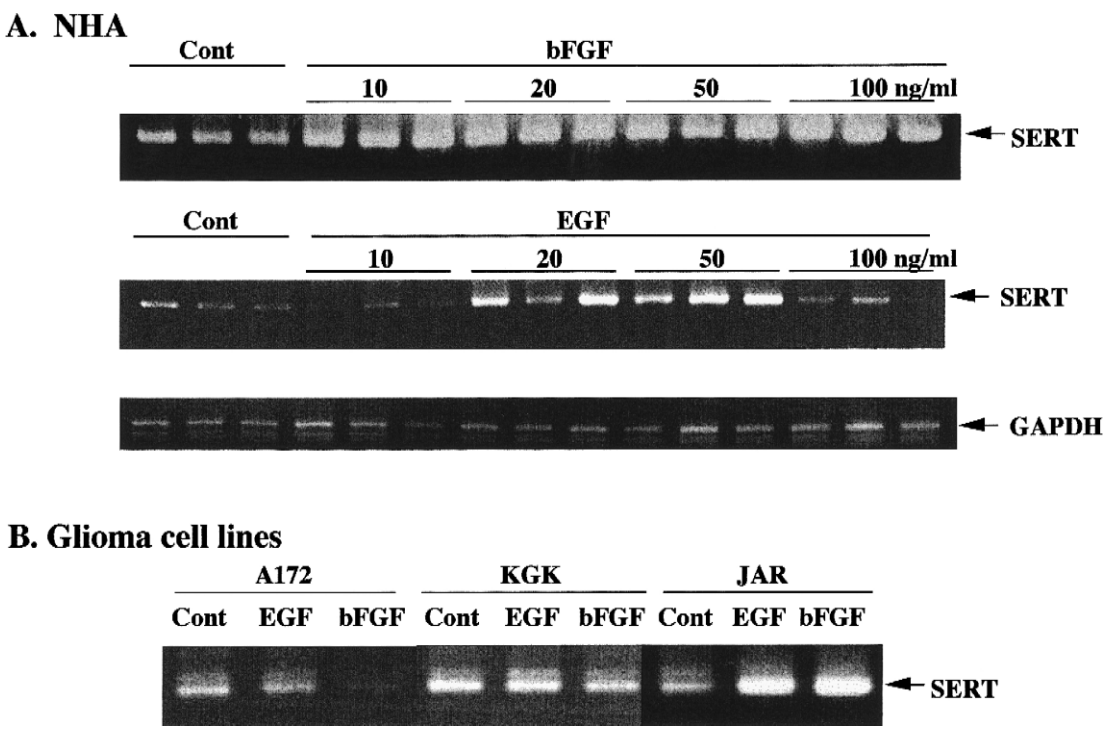

Fig. 4. Effects of EGF or bFGF on the expression of SERT mRNA in normal human astrocytes and human glioma cell lines. (A) Normal human astrocytes (NHA) were treated with vehicle (Cont), EGF or bFGF (10–100 ng/ml) for 2 days. (B) Human glioma cell lines, A172 and KGK, and JAR (human choriocarcinoma cell line) were treated with vehicle (Cont), EGF (20 ng/ml) or bFGF (100 ng/ml) for 2 days. Extracted total RNA was amplified by RT-PCR using the specific primers shown in Table 1. PCR products from three dishes for each treatment in A or from one dish in B were separated by electrophoresis on 3% agarose gels and visualized using ethidium bromide staining.

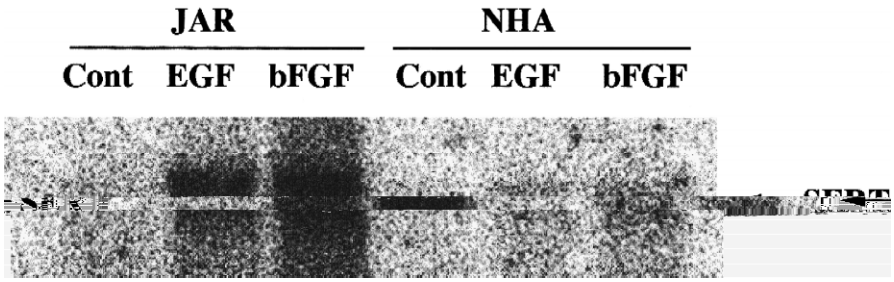

Fig. 5. Northern blotting of SERT mRNA from EGF- or bFGF-treated normal human astrocytes and JAR. Normal human astrocytes (NHA) and JAR were treated with the vehicle (Cont), EGF (20 ng/ml) or bFGF (100 ng/ml) for 2 days. Extracted total RNA (20 μg) was separated by electrophoresis and transferred to a membrane, which was then hybridized with a [^{32}P]-labeled human SERT cDNA probe. mRNA on the membrane was visualized using BAS3000.

terminated by washing the cells three times with ice-cold uptake buffer. The cells were dissolved in 1 ml of 0.1 N NaOH, and aliquots were taken for liquid scintillation counting. Specific uptake was determined by subtracting the uptake in the presence of 1 μM of imipramine (non-specific uptake) from the total uptake.

3. Results

3.1. Monoamine transporter gene expression in normal human astrocytes and human glioma cell lines

We examined the monoamine transporter gene expression by RT-PCR in normal human astrocytes, and a specific RT-PCR product for SERT mRNA (319 bp) was detected, as shown in Fig. 1. The full nucleotide sequence of SERT mRNA determined using 3′ and 5′ RACE was in accordance with the reported sequence of neuronal SERT (data not shown). mRNAs for the other transporters, DAT and NET, were not detected (Fig. 1). Other glioma cell lines, KG-1-C, A172 and KGK are also expressed only SERT mRNA (Fig. 2). Moreover, we examined the expression of SERT protein and GFAP in normal human astrocytes by immunohistochemical staining. Approximately 60% of cultured cells were stained with anti-SERT antibody, and anti-SERT-positive cells were also stained with anti-GFAP (Fig. 3).

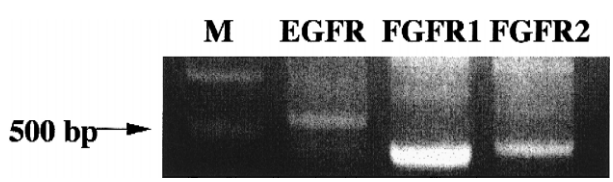

Fig. 6. mRNA expression of EGF or FGF receptors in normal human astrocytes. Extracted total RNA from normal human astrocytes was amplified by RT-PCR using the specific primers for EGFR, FGFR1 and FGFR2 shown in Table 1. PCR products were separated by electrophoresis on 3% agarose gels and visualized using ethidium bromide staining. M —molecular weight marker.

3.2. Effects of bFGF or EGF on the SERT mRNA expression and [^{3}H]serotonin uptake in normal human astrocytes

3.2.1. MRNA expression

bFGF treatment for 2 days markedly increased SERT mRNA expression in normal human astrocytes. The intensity of the RT-PCR product was similar within the concentration range of 10–100 ng/ml. EGF also stimulated SERT mRNA expression, but apparently only at 20 and 50 ng/ml (Fig. 4A). The increase in SERT mRNA in normal human astrocytes after EGF (20 ng/ml) and bFGF (100 ng/ml) stimulation for 2 days was also confirmed by Northern blotting (Fig. 5). In contrast, in glioma cell lines, A172 and KGK, SERT mRNA expression did not change (EGF) or decreased (bFGF) after EGF (20 ng/ml) or bFGF (100 ng/ml) stimulation for 2 days, while in JAR, both growth factors increased SERT mRNA expression

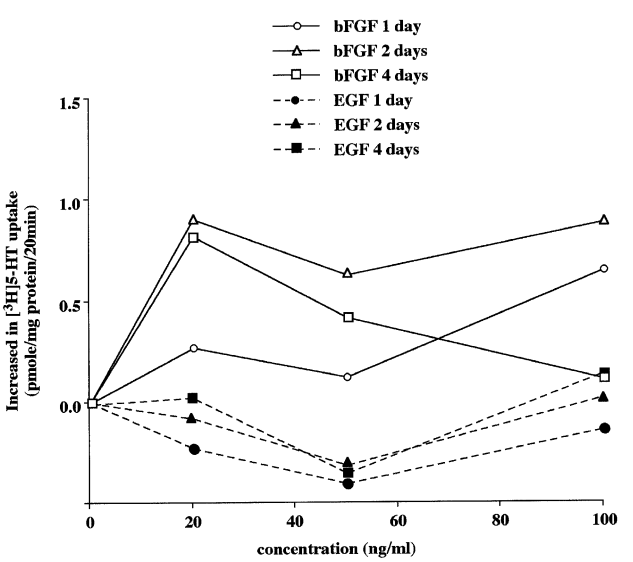

Fig. 7. Effects of EGF or bFGF on [^{3}H]serotonin uptake into normal human astrocytes. Cells were treated with EGF or bFGF (20, 50 and 100 ng/ml) for 1, 2 or 4 days. Subsequently, cells were incubated with [^{3}H]serotonin at 37°C for 20 min. Uptake under basal conditions was negligible and subtracted from that after EGF or bFGF treatment.

(Figs. 4B and 5). mRNAs for the EGF and bFGF receptors, EGFR, FGFR1 and FGFR2, were detected in normal human astrocytes by using RT-PCR (Fig. 6). EGFR and FGFR1 mRNA expression was detected in A172, KGK and JAR, but FGFR2 mRNA expression was detected only in JAR by RT-PCR (data not shown).

3.2.2. [^{3}H] serotonin uptake assay

Under basal conditions, the uptake of [^{3}H]serotonin into normal human astrocytes was almost negligible. However, as shown in Fig. 7, bFGF increased [^{3}H]serotonin uptake in normal human astrocytes in a concentration- and incubation time-dependent manner. Maximal [^{3}H]serotonin uptake was observed at 100 ng/ml after 1 day of stimulation, and at 20 ng/ml after 4 days of stimulation. After 2 days of stimulation, bFGF increased the uptake to a similar extent within the range of 20–100 ng/ml. In contrast, EGF (20–100 ng/ml) did not affect serotonin uptake after either a 1-, 2- or 4-day incubation. Imipramine and sertraline at 10^{-8} M almost completely inhibited serotonin uptake (data not shown). The affinity (K_m) for specific uptake was determined to be 218 ± 13 nM and the maximal velocity (V_{max}) to be 0.034 ± 0.005 pmol/mg protein/min after 2 days of stimulation with 50 nM bFGF. In the case of JAR, both bFGF and EGF increased [^{3}H]serotonin uptake in a concentration-dependent manner (10–50 ng/ml), and uptake was saturated at 20 ng/ml (Fig. 8). The stimulatory effect of bFGF was greater than that of EGF. The increase in serotonin uptake elicited by the growth factors was similar after 1 and 3 days of incubation. In a glioma cell line, A172, neither EGF (20 ng/ml) nor bFGF (100 ng/ml) affected serotonin uptake after 2 days of incubation (data not shown).

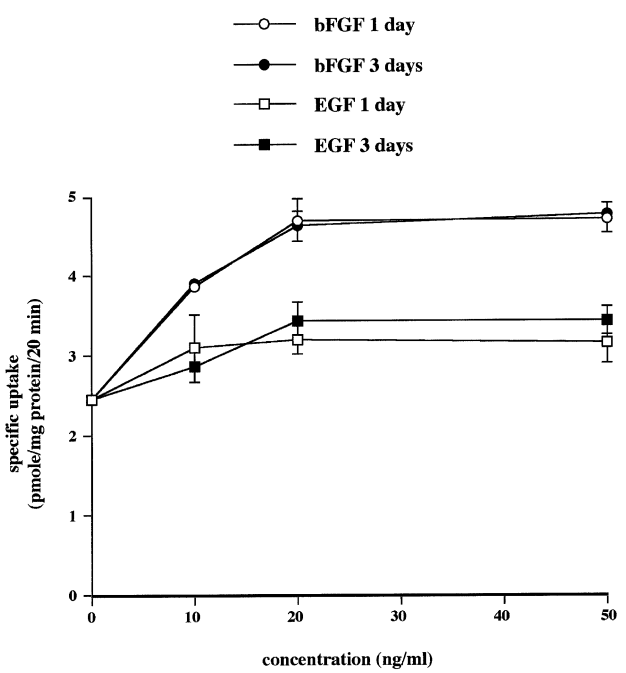

Fig. 8. Effects of EGF or bFGF on [^{3}H]serotonin uptake into JAR. Cells were treated with EGF or bFGF (10, 20 and 50 ng/ml) for 1 or 3 days. Subsequently, cells were incubated with [^{3}H]serotonin at 37°C for 20 min.

4. Discussion

In this study, we attempted to detect the expression of mRNA for the known monoamine transporters (SERT, NET and DAT) in normal human astrocytes and human glioma cell lines using the RT-PCR method. We detected SERT mRNA expression in normal human astrocytes, but neither NET nor DAT was detected. The human glioma cell lines, A172 and KGK, were also found to express SERT mRNA. Furthermore, the sequence of glial SERT mRNA isolated from normal human astrocytes completely corresponded to that of neuronal SERT mRNA. Experiments with double immunofluorescence confirmed that the SERT and GFAP proteins are both expressed in normal human astrocytes. Bel et al. (1997) and Hirst et al. (1998) also showed the expression of part of the neuronal SERT mRNA or SERT protein in rat astrocytes. These findings suggest that identical SERT is expressed, and that serotonin is taken up, in both astrocytes and neurons. The K_m value for serotonin uptake in normal human astrocytes (218 nM) in our study or that in rat astrocytes (380 nM) is a little higher than the value (80 nM) obtained for the synaptosomal rat serotonin transporter (Ross and Renyi, 1975). However, the reason for the difference of affinity in serotonin uptake between astrocytes and neurons is unclear. Recently, the extraneuronal monoamine transporter (EMT) and the organic cation transporter (OCT3), which can transport monoamines, mainly catecholamines, were cloned and considered to be expressed in glial cells (Wu et al., 1998; Gründemann et al., 1998). Thus, glial cells are thought to take up catecholamines through these newly cloned transporters, and serotonin through SERT.

Next, we investigated the effects of stress-related growth factors on SERT mRNA expression. bFGF or EGF increased SERT mRNA expression in normal human astrocytes and JAR in a concentration-dependent manner. The effects of bFGF were greater than those of EGF in normal human astrocytes. Kekuda et al. (1997) also reported that SERT expression increased in EGF-stimulated JAR, probably through tyrosine kinase activation of the EGF receptor. In this study, we detected the expression of FGFR1, FGFR2 (receptors for bFGF) and EGFR (EGF receptor) in normal human astrocytes by RT-PCR. In a preliminary experiment, we found that the promoter activity of the human SERT gene was stimulated by bFGF, as determined with the green fluorescence protein (GFP) expression system (data not shown). Therefore, it is suggested that the increase in SERT mRNA expression is mediated by tyrosine kinase activation of EGF or bFGF receptors and the stimulation of the promoter.

We also found that bFGF, but not EGF, increased specific [^{3}H]serotonin uptake into normal human astrocytes, indicating that bFGF has stimulatory effects on SERT mRNA levels and, subsequently, on its protein synthesis. However, the reason EGF did not stimulate serotonin uptake into normal human astrocytes remains unclear. Inazu et al. (1999b) also reported that bFGF stimulates dopamine uptake in rat astrocytes, suggesting that bFGF might induce several transporters in glial cells. However, neither EGF nor bFGF stimulated SERT mRNA expression in A172 and KGK glioma-derived cell lines. These findings might reflect a failure of the regulation of SERT mRNA expression through EGF- or bFGF-linked signal transduction in glioma cell lines, but the precise mechanisms are not clear.

The association between excessive serotonin and neurodegeneration has been suggested (Joseph et al., 1992). A 5-HT_2 receptor antagonist, ketanserin, proved to be neuroprotective against brain infarction after ischemia in rats (Klisch and Bode-Greuel, 1992). It has been reported that the expression of mRNA for bFGF and the FGF receptor increased in glial cells under conditions of stress, such as ischemia or trauma in the brain (Flinklestein et al., 1988; Takami et al., 1993). In the present study, both SERT mRNA expression and [^{3}H]serotonin uptake were shown to increase in bFGF-stimulated normal human astrocytes and JAR. Taken together with these findings, it is supposed that, during stress in the brain, an increased expression of bFGF and its receptor stimulates SERT expression in glial cells and suppresses serotonin-induced neurotoxicity through increased serotonin uptake. However, further studies are necessary to elucidate the pathophysiological role of bFGF-stimulated SERT expression in glial cells.

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