Localization of fractalkine and CX_3CR1 mRNAs in rat brain: does fractalkine play a role in signaling from neuron to microglia?

Atsushi Nishiyori[a], Masabumi Minami[a], Yoshikazu Ohtani[a], Shinya Takami[a], Junki Yamamoto[a], Nami Kawaguchi[a], Toshiaki Kume[b], Akinori Akaike[b], Masamichi Satoh[a,]*

[a]*Department of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8304, Japan*
[b]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8304, Japan*

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Abstract Localization of the mRNAs for fractalkine, a CX_3C chemokine, and for its receptor CX_3CR1 was investigated in the rat brain. In situ hybridization study revealed that fractalkine mRNA was dominantly expressed in neuronal cells particularly in the olfactory bulb, cerebral cortex, hippocampus, caudate putamen and nucleus accumbens. In vitro study using enriched neuronal or glial culture supported the dominant expression of fractalkine mRNA in neurons. On the other hand, CX_3CR1 mRNA was dominantly expressed in glial cells throughout the whole brain. The in vitro study suggested the cells expressing CX_3CR1 mRNA are microglia, not astrocytes or neurons. Fractalkine appears to function as a signal molecule from neuron to microglia.

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Key words: Fractalkine; CX_3C chemokine receptor; Messenger RNA; Neuron-microglia interaction; Rat brain

1. Introduction

Fractalkine is a novel protein belonging to the fourth class of chemokines [1,2]. It is the only chemokine to possess a CX_3C motif in the chemokine domain as well as a mucin-like domain. Membrane-bound and secreted forms of fractalkine have been reported [1], while no member of the C, CC or CXC chemokine family has a membrane-bound form. [3,4]. Furthermore, the expression of fractalkine mRNA is hardly detectable in T cells, B cells and mononuclear cells [1], whereas abundant expression of C, CC and CXC chemokines is observed in these cells [4–6]. Fractalkine is different from other chemokines also in the expression in the brain. Fractalkine mRNA is constitutively expressed in the human [1] and mouse [2] brain, while the expression of most other chemokines only becomes detectable in the brain under certain pathological conditions, such as in cases of ischemia, viral infection and autoimmune disease [7–9].

Recently, the orphan receptor V28 was identified as the receptor for fractalkine and renamed CX_3C chemokine receptor CX_3CR1, which was reported to have seven putative transmembrane helices characteristic to G protein-coupled receptors and to be expressed in leukocytes and mediate the signals to control their migration and adhesion [10]. Furthermore, the rat orphan receptor RBS11 was shown to function as the receptor for fractalkine in HEK293 cells and *Xenopus* oocytes by Fra 2 assay and electrophysiological analyses [11]. The transcript for CX_3CR1, as well as that for fractalkine, has been shown to express in human [12] and rat [13] brains.

Constitutive expression of the mRNAs for both fractalkine and its receptor, CX_3CR1, in the central nervous system implies that this chemokine has physiological roles there. Alternatively, since chemokines are known to be the molecules which control trafficking of immune cells to inflammation [14], fractalkine may play a critical role in the trafficking of neural cells in pathological conditions. Since this chemokine has been shown to function as an adhesion molecule in vitro [1,10], it may control cell-cell interactions in the brain. In the present study, as a first step to elucidate the function of fractalkine in the central nervous system, we investigated the localization of fractalkine and CX_3CR1 mRNAs in the rat brain using an in situ hybridization technique. In addition, to identify the cellular source(s) of these mRNAs, expression of the mRNAs in enriched culture of neurons, astrocytes or microglia was examined. The results revealed intense expression of fractalkine and CX_3CR1 mRNAs in neurons and microglia, respectively, implicating fractalkine as a signal molecule from neuron to microglia.

2. Materials and methods

2.1. Materials

Proteinase K was purchased from Merck (Darmstadt, Germany). Salmon sperm DNA and ribonuclease A (RNase A) were from Sigma (St. Louis, MO, USA). Yeast tRNA and dispase were from Boehringer Mannheim (Mannheim, Germany). Eagle's minimum essential medium (MEM) was purchased from Nissui (Tokyo, Japan). All other chemicals were from Nacalai Tesque (Kyoto, Japan).

2.2. cDNA cloning for rat fractalkine and CX_3CR1

cDNAs for rat fractalkine and its receptor CX_3CR1 were cloned from whole brains of Sprague-Dawley rats which had been intraperitoneally injected with lipopolysaccharide (1 mg/kg). Total RNA was extracted by a single step acid phenol/chloroform method using ISOGEN (Nippongene, Tokyo, Japan) and used as a template for synthesis of the first-strand cDNA, where 20 μg of total RNA was incubated at 37°C for 1 h with a mixture of 200 U/μl of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, USA), 1× reverse transcription buffer, 10 mM dithiothreitol (DTT), 1 unit/μl RNasin, 10 ng/μl DNA random hexamers and 0.5 mM each of dATP, dGTP, dCTP and dTTP in a volume of 60 μl. An aliquot of the RT product was added to 2 units of Taq DNA polymerase (Perkin-Elmer, Foster City, CA, USA) and 20 pmol each of forward and reverse primers in 50 μl of Taq buffer containin-g 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X100 and 0.2 mM each of dATP, dGTP, dCTP and dTTP. The forward (5′-cctgtagctttgctcatccac-3′) and reverse (5′-cacgggcaccaggacatatga-3′) primers for fractalkine cDNA and the forward ((5′-agctgctcaggacctcaccat-3′) and reverse (5′-gtcatatgcaggaactctggg-3′) primers for CX_3CR1 cDNA were designed on the basis of the sequence of human fractalkine cDNA (GenBank, U84487) and rat CX_3CR1 cDNA, RBS11 (GenBank, U04808) respec-

*Corresponding author. Fax: (81) (75) 753-4586.
E-mail: msatoh@pharm.kyoto-u.ac.jp

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tively. The cDNAs were amplified by 35 cycles of polymerase chain reaction (PCR). The amplified cDNAs were electrophoresed in 1.5% agarose gel, purified by Gene Clean II kit (Bio-101, La Jolla, CA, USA) and ligated to pCR2.1 (Invitrogen, San Diego, CA, USA) or pCR-script-Amp (Stratagene, La Jolla, CA, USA) plasmid vector. Fractalkine cDNA was subcloned to pBluescript plasmid vector (Stratagene). Sequence analyses using Abi Prism dye terminator sequencing ready reaction kit (Perkin-Elmer) confirmed that the obtained CX_3CR1 cDNA contained a full length, but the fractalkine cDNA was only part of the coding region. The full length cDNA for rat fractalkine was obtained by 5′- and 3′-RACE PCR using Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). The reverse primer for 5′-RACE (5′-tcacaccggcaccaggacgtac-3′) and the forward primer for 3′-RACE (5′-gacacctaggatcacctcggccaccagg-3′) were designed on the basis of the sequence of the obtained partial cDNA for rat fractalkine.

2.3. *In situ hybridization histochemistry for fractalkine and CX_3CR1 mRNAs*

Male Sprague-Dawley rats weighing 180–200 g were used. The brains were rapidly removed and frozen in powdered dry ice. Sagittal sections (16 μm) were prepared in a cryostat, thaw-mounted onto gelatin-coated slides and stored at −80°C until in situ hybridization was conducted. In situ hybridization was conducted as described previously [15]. ^{35}S-Labeled antisense and sense RNA probes were synthesized in the presence of [α-^{35}S]UTP (30 TBq/mmol, Amersham, Buckinghamshire, UK) using the linearized plasmids and T7 and T3 RNA polymerases (Promega, USA), respectively. Specific activities of ^{35}S-labeled probes were $1.0–1.2\times10^9$ cpm/μg. After in situ hybridization, the slides were exposed to an autoradiographic film, Hyperfilm βmax (Amersham). After 10 days exposure, the film was developed with D-19 (Kodak, New York, USA). Then, the slides were dipped in autoradiographic emulsion NTB-3 (Kodak) diluted 1:1 with water. After 3 weeks of exposure at 4°C, they were developed with D-19 (Kodak) and counterstained with cresyl violet. Anatomical nomenclature followed Paxinos and Watson [16].

2.4. *Detection of fractalkine and CX_3CR1 mRNAs in the brain and enriched neuronal and glial cultures by RT-PCR*

Sprague-Dawley rat brains were used for neuronal and glial cultures. The cultures were maintained at 37°C in 5% CO_2/95% air. Neuronal culture was prepared from the cerebral cortex of fetal rats (16–18 days gestation) as previously described [17]. The culture was used for the extraction of total RNA after 10–14 days of cultivation. Astrocyte culture was prepared from the cerebral cortex of neonatal rats (1 day old) according to the method of Takuma et al. [18]. The purity of astrocytes was >90%, as determined by immunostaining with anti-glial fibrillary acidic protein antibody. Microglial culture was prepared from whole brains of neonatal rats (1 or 2 days old) according to the method of Suzumura et al. [19]. The purity of microglia was >98%, as determined by immunostaining with OX-42 antibody.

Total RNA was extracted from cultured cells or whole brains by a single step acid phenol/chloroform method. 5 μg of each total RNA sample was used for the synthesis of the first-strand cDNA, then PCR was carried out in 20 μl of reaction mixture containing 0.25 μl of RT product. The conditions for the RT reaction and PCR were the same as described above. The forward (5′-gaattcctggcgggtcagcacctcggcata-3′) and reverse (5′-aagcttttacagggcagcggtctggtggt-3′) primers for rat fractalkine cDNA and the forward (5′-agctgctcaggacctcaccat-3′) and reverse (5′-gttgtggaggccctcatggctgat-3′) primers for rat CX_3CR1 cDNA were designed on the basis of the sequence of rat fractalkine cDNA, which was cloned and sequenced in the present study, and rat CX_3CR1 cDNA (GenBank, U04808), respectively. The cDNAs were amplified by 35 cycles of PCR. Each cycle of PCR for both fractalkine and CX_3CR1 consisted of denaturation at 94°C for 60 s, annealing at 60°C for 30 s and extension at 72°C for 60 s. The PCR products were electrophoresed in 3% agarose gel in TAE buffer, and then the gel was stained with ethidium bromide and photographed. PCR products were confirmed to be the partial cDNAs of fractalkine and CX_3CR1, respectively, by sequence analyses and restriction enzyme digestion.

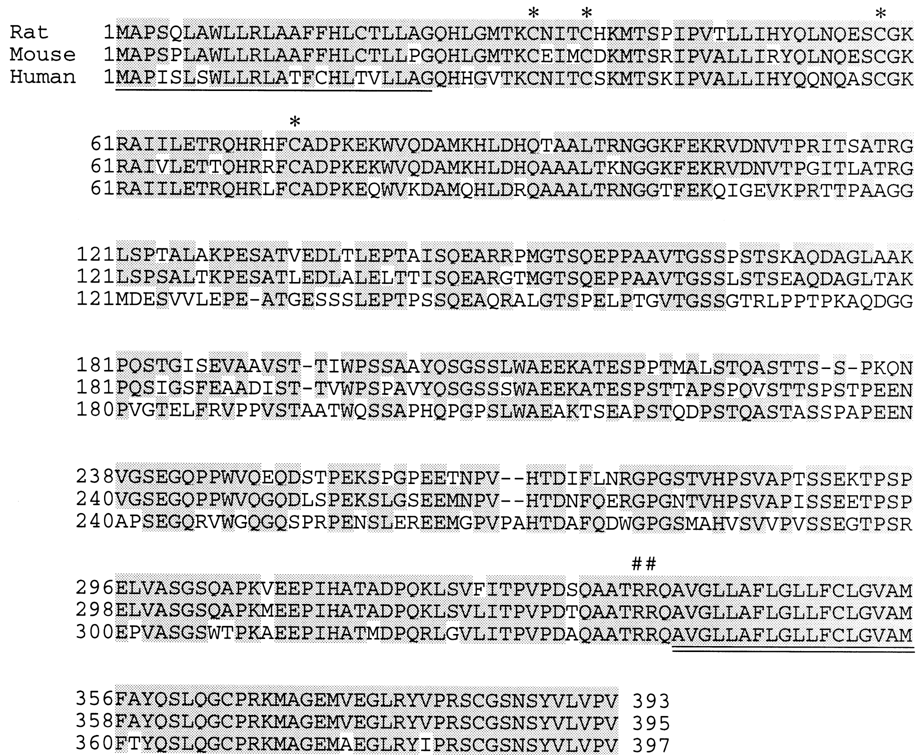

Fig. 1. Deduced amino acid sequence of rat fractalkine compared with mouse and human counterparts. Amino acid residues conserved between more than two species are shadowed. The predicted signal peptide is indicated by an underline, the conserved cysteine residues characteristic of the chemokine superfamily by asterisks (*), and the putative transmembrane domain by a double underline. The dibasic motif (-Arg-Arg-) in the extracellular juxtamembrane, a postulated enzymatic cleavage site, is conserved among the three species and shown by sharps (#).

3. Results

3.1. Rat fractalkine cDNA

A partial cDNA (130–1174) of the coding region of rat fractalkine was cloned from rat whole brain by a RT-PCR method, and then the remains (−17–129 and 1180–1224) were cloned by 5′-RACE and 3′-RACE methods. Five dependent clones were sequenced to avoid errors due to misincorporation by Taq DNA polymerase which lacks 3′-5′ exonuclease (proofreading) activity. The deduced amino acid sequence of rat fractalkine was compared with that of mouse and human fractalkine (Fig. 1). Rat fractalkine shares 84.6% and 66.1% homology overall, 85.5% and 82.7% homology in the chemokine domain, 79.9% and 53.9% homology in the mucin-like domain, 100% and 100% homology in the transmembrane domain, and 100% and 91.9% homology in the intracellular region, with mouse and human counterparts, respectively. The CX_3C motif in the chemokine domain and the dibasic motif (Thr-Arg-Arg-Gln) in the extracellular juxtamembrane are conserved among these three species.

3.2. Expression of fractalkine mRNA in the rat brain

In situ hybridization study revealed that fractalkine mRNA was expressed in various brain regions. Fractalkine mRNA was expressed intensely in the olfactory bulb, cerebral cortex, hippocampus, caudate putamen and nucleus accumbens, weakly in the hypothalamus and midbrain, but scantly in the pons, medulla oblongata and cerebellum (Fig. 2a). In white matter, such as corpus callosum, expression of the mRNA was not detected. Sense RNA probe did not detect any significant signals in the adjacent section (Fig. 2b). Microautoradiograms showed that the signals were dominantly localized to the cells which had relatively large and lightly counterstained nuclei, likely neuronal cells, in all regions where fractalkine mRNA was expressed (Fig. 2c).

3.3. Expression of CX_3CR1 mRNA in the rat brain

CX_3CR1 mRNA was expressed throughout the whole brain almost uniformly (Fig. 2d). Different from fractalkine mRNA, CX_3CR1 mRNA was expressed in white matter as intensely as in gray matter. Slightly more intense signals were observed in the choroid plexus and substantia nigra. Sense RNA probe did not detect any significant signals in the adjacent section (Fig. 2e). Throughout the brain excluding the choroid plexus, the signals for CX_3CR1 mRNA localized to the cells which had relatively small and densely counterstained nuclei, likely glial cells (Fig. 2f).

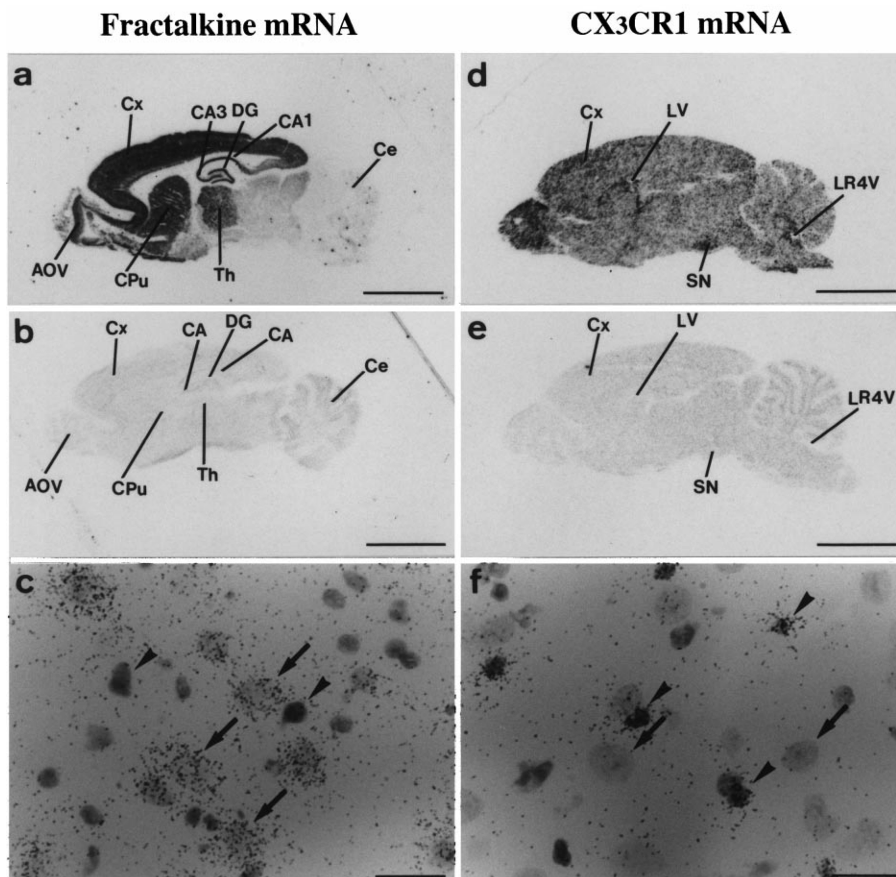

Fig. 2. Localizations of fractalkine (a) and CX_3CR1 (d) mRNAs in the rat brain as visualized by in situ hybridization with ^{35}S-labeled antisense RNA probes. Sense RNA probes did not give significant signals (b, e). Microautordiograms for the expression of fractalkine (e) and CX_3CR1 (f) mRNAs in the cerebral cortex. Fractalkine mRNA was localized to the cells which had relatively large and lightly counterstained nuclei, likely neuronal cells, shown by arrows (c). On the other hand, CX_3CR1 mRNA was expressed in the cells which had relatively small and densely counterstained nuclei, likely glial cells, shown by arrowheads (f). AOV, anterior olfactory nucleus; Cx, cerebral cortex; CA1, CA1 part of the Ammon's horn; CA3, CA3 part of the Ammon's horn; CPu, caudate putamen; DG, dentate gyrus; Th, thalamus; Ce, cerebellum; SN, substatia nigra; LV, lateral ventricle; LR4V, lateral recess 4th ventricle. Bar = 5 mm in a, b, d and e, and 25 μm in c and f.

3.4. Expression of fractalkine and CX_3CR1 mRNAs in enriched neuronal and glial cultures

To clarify which types of cell express fractalkine and CX_3CR1 mRNAs, the expression of these mRNAs in enriched neuronal and glial cultures was investigated by a RT-PCR method. RT-PCR with the samples from whole brain, which had been shown to contain both fractalkine and CX_3CR1 mRNAs by in situ hybridization, gave a significant single band for each mRNA with the predicted mobility in ethidium bromide-stained agarose gel. Primary cerebral cortical neurons as well as whole brain intensely expressed fractalkine mRNA (Fig. 3a). In addition, fractalkine mRNA was weakly expressed in microglia and astrocyte cultures. On the other hand, CX_3CR1 mRNA was expressed dominantly in microglia (Fig. 3b), though the expression was not detected in cerebral cortical neurons and astrocytes.

4. Discussion

In this study, we isolated rat fractalkine cDNA and determined its nucleotide sequence. The deduced amino acid sequence of rat fractalkine shared 84.6% and 66.1% identity overall with human and mouse counterparts, respectively. Higher homology was observed in the chemokine domain. The amino acid sequences in the transmembrane and intracellular domain were almost completely conserved among these three species. The dibasic motif (Thr-Arg-Arg-Gln) in the extracellular juxtamembrane was conserved, suggesting that rat fractalkine is also released by enzymatic cleavage at this site [20].

Next, we elucidated the localization of the mRNAs for fractalkine and its receptor CX_3CR1 in the rat brain. Fractalkine mRNA was constitutively expressed in normal rat brain. Fractalkine mRNA was expressed intensely in most parts of the telencephalon and some parts of the diencephalon, but only weakly or scantly in the rest of the brain. No signals for fractalkine mRNA were observed in white matter. The signals were shown to be localized to cells which had relatively large nuclei counterstained lightly by cresyl violet. These findings suggest that fractalkine mRNA is expressed in neurons. This idea is supported by the experiments using enriched neuronal and glial cultures, which showed that fractalkine mRNA was expressed intensely in cerebral cortical neurons, but very weakly in enriched microglia and astrocyte cultures. These characteristics of the expression of fractalkine mRNA in the brain are unique, because the mRNAs for most members of the C, CC or CXC chemokine family cannot be detected in normal brain. Furthermore, although the mRNAs for some CC and CXC chemokines are reported to be expressed inducibly in the brains under certain pathological conditions, in such cases the mRNAs are expressed dominantly in glial cells, not in neurons [7–9]. Fractalkine mRNA was expressed very weakly or scantly in the pons, medulla oblongata and cerebellum, while CX_3CR1 mRNA was expressed in these brain regions as intensely as in other regions, such as the cerebral cortex, hippocampus and caudate putamen. These findings suggest a possibility that other CX_3C chemokines than fractalkine are expressed in the brain. This possibility is seemed to be likely, since most CCR- and CXCR-type chemokine receptors are known to have two or more chemokines as their ligands.

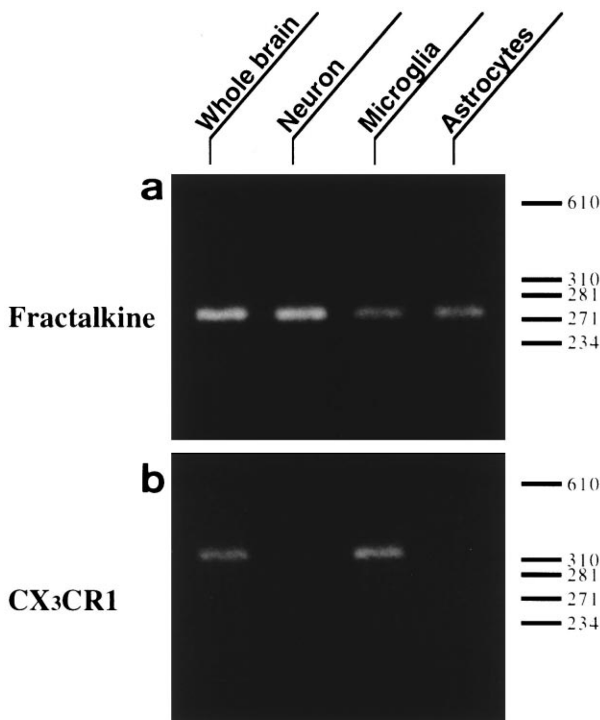

Fig. 3. Detection of fractalkine (a) and CX_3CR1 (b) mRNAs in the brain and enriched neuronal and glial cultures by a RT-PCR method. The mobility of size markers is indicated on the right.

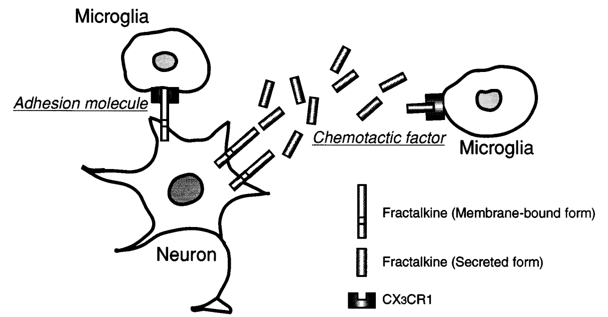

Fig. 4. Hypothetic roles of fractalkine as both a 'chemotactic factor' and an 'adhesion molecule' in the interaction between neuronal and microglial cells. The secreted form of fractalkine released from neurons may function as a chemotactic factor to cause the activation and migration of microglial cells. On the other hand, fractalkine in membrane-bound form could play a role as an 'adhesion molecule' in direct interaction between neuronal and microglial cells, such as synaptic stripping, and be involved in synaptic reorganization in normal and/or degenerated brains.

CX_3CR1 mRNA was expressed throughout the whole brain almost uniformly. Also, it was expressed in white matter as intensely as in gray matter. The signals for CX_3CR1 mRNA were localized to the cells which had relatively small nuclei counterstained densely by cresyl violet. These findings suggest that CX_3CR1 mRNA is expressed in glial cells rather than neurons. The results from the experiments using enriched neuronal and glial cultures indicate that the mRNA for this receptor is dominantly expressed in microglial cells. To confirm the existence of functional CX_3CR1 receptors exclusively in microglia, not in neurons and astrocytes, we are currently examining the effects of fractalkine on intracellular Ca^{2+} concentration in these cells. Microglia are reported to express a

variety of chemokine receptors, such as CCR3, CCR5, CXCR4 [21,22], which are thought to mediate the signals involved in the activation and/or migration of microglia. CX_3CR1 might also play a crucial role in regulating the activation and/or migration of microglia in the brain. In addition to the brain parenchyma, CX_3CR1 mRNA was expressed in the choroid plexus. This result is interesting, when considering that the choroid plexus is located at the boundary between brain parenchyma and peripheral circulation. Further experiments, including the identification of cell species, are necessary to clarify the roles of CX_3CR1 receptors expressed in the choroid plexus.

The present study demonstrated that, unlike other chemokines, fractalkine is constitutively expressed in neurons of normal rat brain. Furthermore, its receptor has been shown to be expressed dominantly in microglia. From these findings, it is conceivable that fractalkine in the brain works as a molecule signaling from neuron to microglia. It is well known that activated microglia are observed around degenerated neurons in brains undergoing ischemia [23] and neurodegenerative disease, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis [24–26]. It has also been reported that axotomy of the rat facial nerve leads to mitotic divisions of microglial cells in the central nucleus of origin [27]. In those cases, signal molecule(s) is thought to be released from the degenerated or damaged neurons to inform microglia of their state. Fractalkine might be released from neurons and function as a chemotactic factor to cause the activation and migration of microglial cells. In fact, in vitro experiments investigating the effects of fractalkine on microglia revealed that it was a potent chemoattractant for microglia [28]. Fractalkine has a membrane-bound form besides a secreted form, while all other chemokines exist as only a secreted form. Furthermore, recently, this chemokine was shown to function as an adhesion molecule [1,10]. Thus, fractalkine could play a role in the cell-cell interaction which needs direct contact between neuronal and microglial cells. One example of such an interaction, in the rat facial nerve model, is 'synaptic stripping', whereby microglia displace synaptic terminals from the surface of regenerating neurons [27]. Since fractalkine is constitutively expressed in the brain, it may be involved in synaptic reorganization for regulating neuronal plasticity not only in degenerated, but in normal brain. Fig. 4 schematically shows the hypothetic roles of fractalkine as both a 'chemotactic factor' and an 'adhesion molecule' in the interaction between neuronal and microglial cells.

Fractalkine immunoreactivity was found to be localized to microglial and endothelial cells in the mouse brain by immunohistochemistry with a polyclonal antibody against the synthesized oligopeptide derived from the chemokine domain of mouse fractalkine [2]. This finding appears to be inconsistent with our present demonstration that fractalkine mRNA was dominantly expressed in neurons. There are several explanations for this discrepancy. (1) A species difference between mouse and rat. (2) Fractalkine mRNA is translated into protein inefficiently in neuronal cells, but efficiently in microglia where weak expression of fractalkine mRNA was detected by a RT-PCR method. (3) In neuronal cells, fractalkine is released immediately after it is produced, since it is thought that fractalkine in membrane-bound form can be enzymatically cleaved to obtain the secreted form. In this case, it is speculated that neurons always act on the surrounding microglia through fractalkine in normal brain because its receptor is constitutively expressed in microglia. Fractalkine immunoreactivity detected on microglia in mouse brains may be due to the fact that fractalkine originally produced in neurons is taken up by microglia by a certain mechanism, such as endocytosis. (4) The chemokine domain of membrane-bound fractalkine expressed on neurons may be masked. This hypothesis is attractive because it implies that the biological activity of fractalkine is regulated by masking and unmasking in response to the state of neuronal cells and/or the condition of the extracellular microenvironment.

In this article, we propose that fractalkine plays a role in the signaling from neuron to microglia in the brain. However, it remains unclear how the synthesis and secretion of fractalkine are regulated and what the physiological and pathological roles of this chemokine in the brain are. As one way to address these issues, we are now raising antibodies against the respective domains of rat fractalkine, that is, the chemokine, mucin-like, transmembrane and intracellular domains.

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