Neuronal expression of fractalkine in the presence and absence of inflammation

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Abstract Fractalkine is the only as yet known member of a novel class of chemokines. Besides its novel Cys-X-X-X-Cys motif, fractalkine exhibits features which have not been described for any other member of the chemokine family, including its unusual size (397 amino acids human, 395 mouse) and the possession of a transmembrane anchor, from which a soluble form may be released by extracellular cleavage. This report demonstrates the abundant mRNA and fractalkine protein expression in neuronal cells. The neuronal expression of fractalkine mRNA is unaffected by experimentally induced inflammation of central nervous tissue.

Key words: Fractalkine; Experimentally induced allergic encephalomyelitis; Inflammation; Neuronal expression

1. Introduction

Chemokines are small secreted proteins that stimulate the directional migration of leukocytes and mediate inflammation [1]. Most recently, a novel subclass of chemokines, the δ-chemokines, was defined, characterised by a Cys-X-X-X-Cys motif [2,3]. So far the only member of this subclass is fractalkine [2]. The murine homologue of human fractalkine was subsequently published by another group and termed neurotactin [3]. Fractalkine exists possibly in two forms: (i) as a type 1 membrane protein furnished with a transmembrane anchor and a cytoplasmic domain and (ii) as a shed glycoprotein of 95 kDa. Both reports demonstrated the chemotactic activity of the shed protein on leukocytes [2,3]. An orphan G-protein-coupled 7-transmembrane receptor -V28- [4] was identified to be a specific receptor for fractalkine and is now termed CX3CR1 [5,6]. Bazan et al. underlined the potential role of fractalkine as an adhesion molecule in leukocyte trafficking at the endothelium and demonstrated that the expression of fractalkine can be induced in cultured human umbilical vein endothelial cells by IL-1β and TNF-α [2]. An immunohistochemical analysis on mouse brain sections reported by Pan et al. [3] implied that microglial cells are the major cellular source of fractalkine in the brain and that protein expression is upregulated by lipopolysaccharide treatment as well as in experimentally induced allergic encephalomyelitis (EAE). This report demonstrates that neuronal cells are the major source of fractalkine biosynthesis in the central nervous system (CNS). Using rat and mouse models of EAE, we show that the high abundance of fractalkine mRNA in neuronal cells and the plasticity of expression are not affected by EAE brain inflammation. Immunohistochemical analysis of human and monkey CNS tissues shows a prominent neuronal staining for fractalkine.

2. Materials and methods

2.1. Models of experimental autoimmune encephalomyelitis

Myelin oligodendrocyte glycoprotein (MOG)-specific T cell lines were derived from Dark Agouti (Harlan Winkelmann, Germany) rats immunised with a synthetic peptide corresponding to the immunodominant T-cell epitope of MOG (amino acids 93–110; EGGYTCFFRDHSYQEEAA) [7]. EAE was induced in naive female recipients (115–150 g) using a dose of 5×10^6 freshly activated T cell blasts. The cells were injected intraperitoneally in a volume of 1 ml culture medium. EAE was induced in female SJL/J mice (Harlan Winkelmann, Germany) by active immunisation with recombinant rat MOG as described previously [8]. Disease was scored on the following scale: 0.5, partial loss of tail tone; 1, complete tail atony; 2, hind limb weakness; 3, hind limb paralysis; 4, moribund; 5, dead.

2.2. Radioactive and non-radioactive in situ hybridisation

For in situ hybridisation, 14 μm thick tissue sections were cut from frozen brains, fixed in phosphate-buffered formaldehyde and processed as previously described [9]. ^{35}S-Labeled cRNA was transcribed from a 396 bp subfragment of rat fractalkine (EMBL Nucleotide Sequence Database accession number Y16813) in antisense and sense orientation. A mouse fractalkine cDNA template of 948 bp was amplified by RT-PCR from total brain mRNA using the following primer set derived from the published sequence [3]: 5′ CTG CCG GGT CAG CAC CTC GGC ATG 3′ and 5′ CCT TGT GGC TGC CTG GGT GTC GGG 3′. Sequence analysis revealed full identity with the published nucleotide sequence. ^{35}S-Labeled cRNA was transcribed from this fragment in antisense and sense orientation. After application of specific ^{35}S riboprobes radioactive signals were detected by autoradiography on Hyperfilm β-max (Amersham) (exposure time 2 days) or coating sections with Kodak NTB-2 nuclear emulsion [10]. For visualisation of non-radioactive hybrids the digoxigenin detection method (Boehringer, Mannheim, Germany) was applied [9]. Dark- and brightfield analysis was performed with an AX70 microscope (Olympus).

2.3. Immunohistochemistry

Immunohistochemistry for fractalkine was carried out on deparaffinised tissue sections using an antigen retrieval technique [10] (10 min cooking in 10 mM Na-citrate buffer, pH 6.0 at 95°C) and the standard avidin-biotin-peroxidase complex technique. Light microscopic immunohistochemistry was performed on deparaffinised sections of Bouin Hollande fixed tissue as described previously [10].

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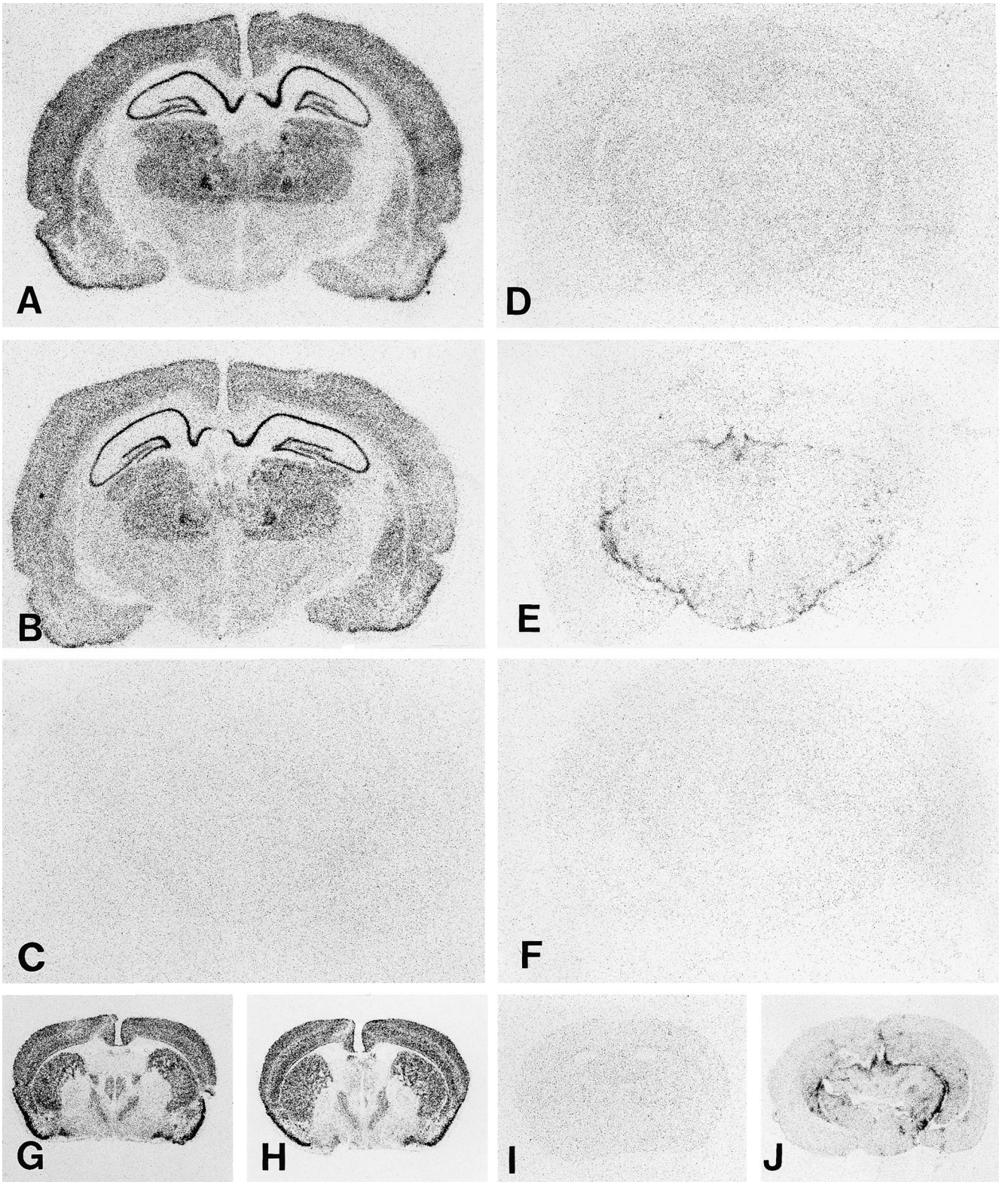

Fig. 1. Species-specific fractalkine antisense cRNA probes were hybridised to cryostat sections of normal rat brain (A) and EAE rat brain (B, clinical stage 3.7) as well as normal mouse brain (G) and EAE inflamed mouse brain (H). X-ray film analysis reveals that fractalkine mRNA is abundantly expressed in many brain regions with high concentrations of neurones (A, B) and (G, H). Comparing sections from normal rat brains (A) and EAE rat brains (B) as well as normal mouse brains (G) and EAE mouse brains (H), no difference in the distribution pattern of fractalkine mRNA is seen. EAE brain inflammation was monitored on consecutive sections using the expression of mRNA coding for the B-chain of rat C1q as a marker for microglial activation as previously described [14]. In non-inflamed brain tissue C1q mRNA is expressed at low abundance (D) whereas C1q mRNA levels are markedly higher in (E), a consecutive section of the EAE inflamed brain hybridised in parallel with fractalkine antisense cRNA (B). No hybridisation signals were obtained with rat fractalkine (C) and mouse fractalkine cRNA probes transcribed in sense orientation (I). A consecutive section of the EAE mouse brain shown in (H) was hybridised with a mouse C1q B-chain specific ^{35}S-labeled cRNA transcribed from a 247 bp long 5′-specific *Bam*HI cDNA subfragment [16] in antisense orientation (J).

2.4. Northern blot analysis

Approximately 15 μg of total RNA isolated by cesium chloride density centrifugation was separated, blotted to a nylon membrane (for method see [11]) and subsequently hybridised with random primed (Boehringer, Mannheim, Germany) [^{32}P]dCTP (Amersham)-labeled cDNA probes for rat fractalkine and the rat B-chain for C1q [11]. Variations in the loading of total RNA were determined by hybridisation with an oligonucleotide specific for the 28S rRNA [12]. The hybridisation signals obtained were quantified by laser densitometry of the autoradiographs using an Ultroscan XL Enhanced Laser Densitometer from Pharmacia Biosystems (Uppsala, Sweden) and normalised against the 28S rRNA abundance (not shown).

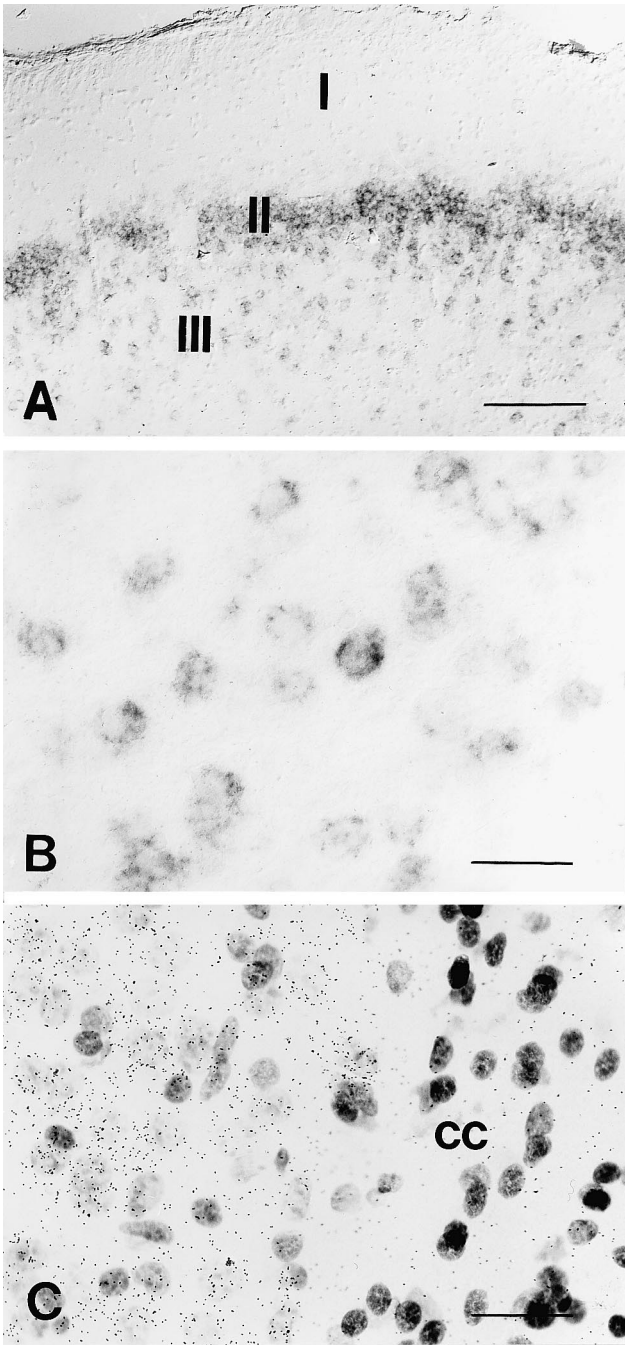

Fig. 2. Using a non-radioactive in situ hybridisation technique fractalkine mRNA was seen in the neuronal layers II and III of piriform cortex (A). Note the absence of any positive labeling in layer I (A) where cell bodies of macro- and microglia predominate. High resolution micrographs of non-radioactive (B) and radioactive (C) in situ hybridisation demonstrate neuronal perikarya labeled for fractalkine mRNA in the layer III of piriform cortex (B) and in the deep layers of parietal cortex (C) while the cells in the white matter of the corpus callosum (cc in C) lack positive labeling. Exposure times of autoradiographs 3 weeks (C); size bars: 200 μm (A), 50 μm (B,C).

3. Results and discussion

To assess whether fractalkine mRNA expression could serve as a marker for microglial cells or even microglial activation, we isolated cDNAs specific for rat and mouse fractalkine for in situ hybridisation and Northern blot analyses of normal and EAE mouse and rat brains [7,8,13]. The results were consistent in both animal models: Fig. 1A depicts fractalkine mRNA expression on a cryostat section of a normal rat brain in comparison to a rat brain with full-blown EAE (Fig. 1B), and the sense control (Fig. 1C). Likewise, no difference in mRNA abundance and the plasticity of expression can be observed between normal mouse brain (Fig. 1G) and EAE inflamed mouse brain (Fig. 1H). Fractalkine mRNA is expressed in brain grey matter with predominance to areas

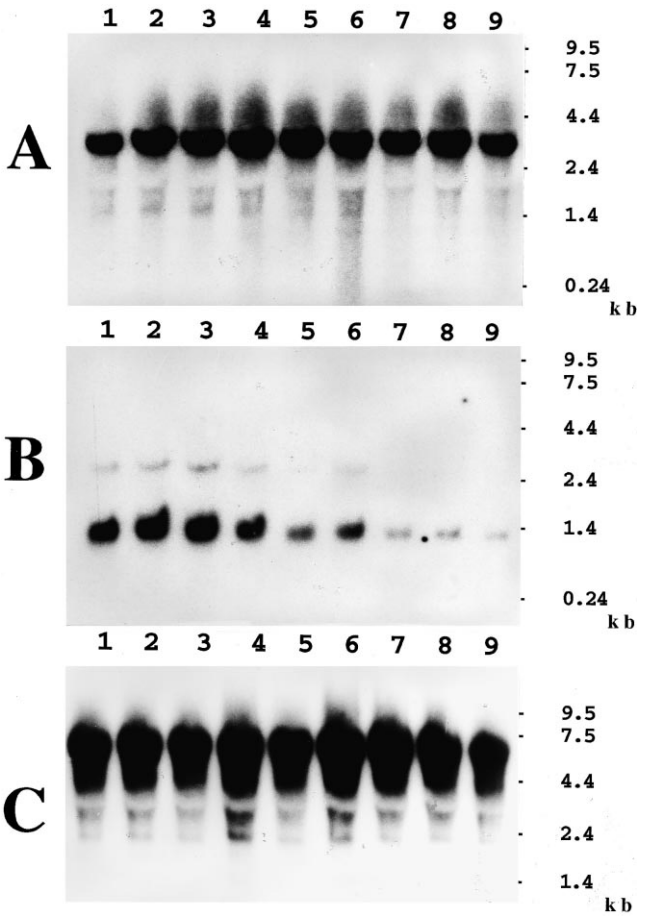

Fig. 3. Total RNA preparations from normal and EAE brains were analysed by Northern blotting and hybridised subsequently with ^{32}P-labeled cDNA probes for rat fractalkine (A) and the rat B-chain for C1q (B, [11]). The latter hybridisation allowed us to monitor the degree of EAE brain inflammation over the time course of EAE induction with animals 7 and 8 on day 2 after T-cell transfer, animals 5 and 6 on day 3 (clinical stage 2), animals 3 and 4 on day 4 (clinical stage 3.2 and 3.4) and animals 1 and 2 on day 5 (clinical stage 3.5 and 3.3 respectively). The 28S rRNA hybridisation signals used to normalise the loading of total RNA are shown in (C).

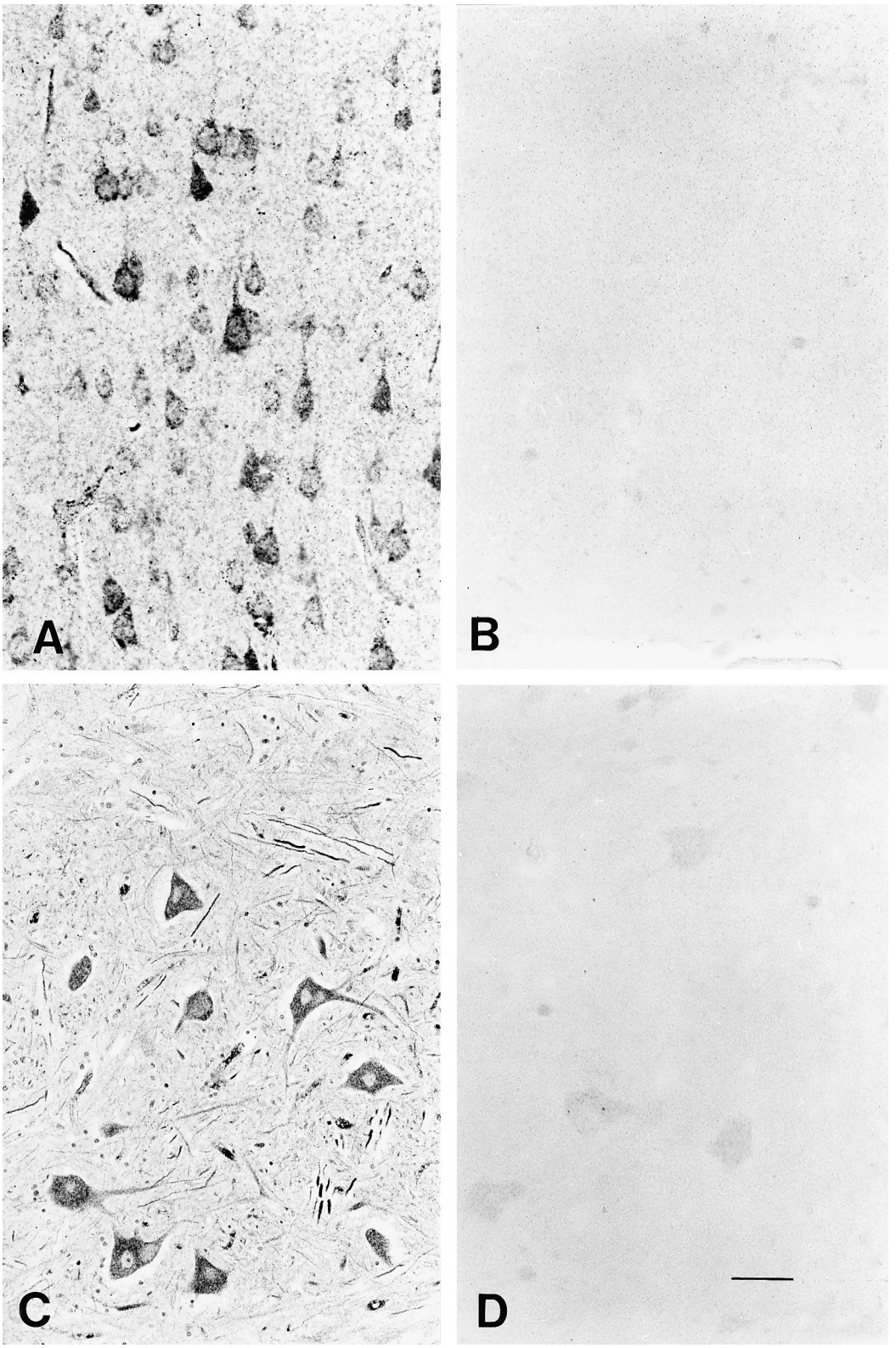

Fig. 4. Immunohistochemistry shows that fractalkine immunoreactivity is present in neuronal cell bodies of rhesus monkey neocortex (A) and in motoneurones and nerve fibres of the ventral human spinal cord (C). The absence of staining in preabsorption controls with excess recombinant fractalkine protein on consecutive sections demonstrates the specificity of the neuronal immunostaining. Size bar: 200 μm.

with high concentration of neurones such as the neocortex and the hippocampal formation and is virtually absent from white matter.

High resolution in situ hybridisation revealed localisation of fractalkine mRNA in neuronal cell bodies with no obvious expression in glial or endothelial cells (Fig. 2A–C). These in vivo results are supported by the detection of fractalkine mRNA in the human neuroblastoma cell lines LAN-1 and

IMR-32 (not shown). EAE did not affect the neuronal pattern of expression or the abundance of fractalkine mRNA as shown in Fig. 1B. The latter observation was confirmed by the Northern blot results shown in Fig. 3A. In all brains, fractalkine mRNA was highly expressed and no differences in the intensity of hybridisation signals were observed between normal (lane 9) and brains at different stages of EAE (lanes 8–1). The same blot was rehybridised with a cDNA probe for the B-chain of rat C1q [11] to verify the course and degree of EAE inflammation in these animals (Fig. 3B) as the severity of disease is paralleled by a dramatic increase in C1q mRNA abundance [14].

Using a polyclonal antibody raised against the recombinant chemokine domain of human fractalkine, it is shown that also fractalkine immunoreactivity is predominant in neurones of rhesus monkey neocortex (Fig. 4A) and in motoneurones of human spinal cord (Fig. 4C).

The finding of a constitutive and abundant neuronal mRNA expression for fractalkine is in agreement with two recently published reports [15,16]. This contribution, however, demonstrates for the first time that (i) fractalkine protein is predominantly expressed in neuronal cells (on human and monkey CNS tissues) and that (ii) fractalkine mRNA abundance in rat and mouse brain tissues is unaffected by EAE brain inflammation.

These results favour the view of more general physiological functions of fractalkine such as the mediation of adhesion and cell-cell communication between neurones and CX3CR1 bearing cells occurring also in the total absence of pro-inflammatory conditions.

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