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Upregulation of chemokine receptor gene expression in brains of Borna disease virus (BDV)-infected rats in the absence and presence of inflammation

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Infection of adult rats with Borna disease virus (BDV) causes CD8 T cell-mediated meningoencephalitis. Previously, we described a complex pattern of chemokine gene expression in the central nervous system (CNS) of such rats. We now found that expression of chemokine receptor genes CXCR3, CCR5, CX_3CR1, and CXCR4 was also upregulated, which is in agreement with the predominance in brains of adult infected rats of T cells and monocytes/macrophages that express these receptors. In contrast to these rats, neonatally infected rats (designated PTI-NB) develop a persistent CNS infection associated with neurodegenerative processes in the absence of inflammation. In brains of PTI-NB rats, sustained expression of chemokines also takes place. We therefore analyzed mRNA expression of selected chemokine receptor genes, as well as of the chemokine fractalkine in brains of PTI-NB rats. We observed a marked increase of CCR5 and CX_3CR1 transcripts in brains of these rats. CX_3CR1 expressing cells were predominantly microglia, and upregulation of CX_3CR1 was mainly due to an increase in the number of CX_3CR1 expressing microglia. Fractalkine gene expression was found to be reduced to similar extents in brains of adult and newborn infected rats. These findings might be of relevance with respect to the selective neuronal cell loss observed in brains of PTI-NB rats. *Journal of NeuroVirology* (2002) **8,** 168–179.

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Introduction

Entry of leukocytes into the central nervous system (CNS) is a crucial event in the pathogenesis of virus-induced, immune-mediated CNS disease (Asensio and Campbell, 2001). Chemokines have been recognized as the key mediators of cerebral leukocyte extravasation and accumulation (Huang *et al*, 2000). The specificity of chemokines is mediated by binding to their respective receptors expressed on different immune cell subsets. So far, the growing family of chemokines can be divided into four subfamilies, based on the position of the first two amino-terminal cysteine residues as well as based on genetic and functional similarities (Baggiolini *et al*, 1997; Bacon *et al*, 1998).

Notably, there is considerable promiscuity of many chemokines and chemokine receptors. While many CXC-chemokines, such as KC and MIP-2 (the rat homologues of human Groα and Groβ, respectively) act on neutrophils (via CXCR2), others primarily act on lymphocytes and monocytes, such as SDF-1 [via CXCR4 (Bleul *et al*, 1997)] or on activated T cells, NK cells, and probably also on monocytes, such as

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IP-10 [via CXCR3 (Taub *et al*, 1993; Loetscher *et al*, 1996)]. Most CC-chemokines, such as MIP-1β, (which binds to CCR5 and CCR8), RANTES (which acts via CCR1, CCR3, and CCR5), and MCP-1 (which binds to CCR2 and CCR4), attract monocytes, but also T cells and NK cells. Lymphotactin, the lone member so far of the C-chemokine family, exclusively attracts lymphocytes. Fractalkine, hitherto the only representative of the CX_3C-chemokine family, is a membrane-bound chemokine, which can be secreted as a soluble chemokine following proteolytic cleavage (Bazan *et al*, 1997). Besides being involved in leukocyte adhesion and attraction, fractalkine has been implicated to be an important mediator of neuron-microglial communication in the CNS due to its high constitutive neuronal expression and the presence of its receptor, CX_3CR1, on microglia (Pan *et al*, 1997; Harrison *et al*, 1998; Nishiyori *et al*, 1998). Constitutive expression of several other chemokine receptor genes also has been shown on CNS resident cells, and accumulating evidence suggests that chemokines not only are involved in leukocyte recruitment, but also seem to play a role in neuronal migration and signaling, neuron-microglial communication, and neurotransmitter release (Asensio and Campbell, 1999; Mennicken *et al*, 1999; Bacon and Harrison, 2000; Bajetto *et al*, 2001). Furthermore, it appears conceivable that unphysiological levels of chemokines and/or their receptors might also result in, or contribute to, CNS pathology (Xia *et al*, 2000; Kaul and Lipton, 1999; Goldberg *et al*, 2001).

Experimental infection of rats with BDV provides a valuable model to study both virus-induced immune-mediated CNS disease, as well as virus-induced neurodevelopmental and behavioral disturbances in the CNS (see Jordan and Lipkin, 2001, and Carbone *et al*, 2001, for reviews). BDV is a noncytolytic, negative-stranded RNA virus with strong neurotropism *in vivo*. In nature, BDV primarily infects horses and sheep where it can induce lethal meningoencephalitis, called Borna disease (BD). Experimental intracerebral (i.c.) infection of adult rats leads to immune-mediated neurobehavioral disease with high mortality. BD in rats is primarily dependent on the action of cytotoxic CD8 T cells (Sobbe *et al*, 1997). Neonatal infection of the rat causes lifelong virus persistence in the absence of gross inflammation (Hirano *et al*, 1983; Carbone *et al*, 1991). These rats, designated as PTI-NB (persistent tolerant infection of the newborn) display distinct neurobehavioral, physiological and neuroanatomical disturbances, such as cerebellar hypoplasia and selective loss of Purkinje, granule, and GABAergic pyramidal neurons in the cerebellum, hippocampus, and frontal cortex, respectively (Eisenman *et al*, 1999; Gonzalez-Dunia *et al*, 2000; Carbone *et al*, 2001). The molecular mechanisms underlying these defects are yet unknown. Interference of BDV with neurotrophin actions as well as increased levels of proinflammatory cytokines possibly play a role (Hornig *et al*, 1999; Sauder and de la Torre, 1999; Zocher *et al*, 2000; Hans *et al*, 2001). The sustained astrocytosis and microglial activation taking place in the PTI-NB rat brain might be the central event leading to BDV-induced disturbances (Sauder and de la Torre, 1999; Weissenböck *et al*, 2000).

Previously, we have described a complex pattern of chemokine gene expression in brains of adult rats following intracerebral BDV infection (Sauder *et al*, 2000). Given, that the specific composition of cerebral immune cell infiltrates is determined by the chemokines expressed at the site of infection, we hypothesized that chemokine receptor gene expression should match the specific chemokines expressed in the infected CNS. Here we describe the increased expression of chemokine receptor genes CXCR3, CCR5, CX_3CR1, and CXCR4 in brains of adult infected rats, which fits well with our hypothesis. We previously not only showed chemokine gene expression in brains of these rats, but also in brains of PTI-NB rats (Sauder *et al*, 2000). This prompted us to speculate that chemokines might be involved in CNS pathology in the absence of immune cell infiltration. To learn more about possible implications of chemokine and chemokine receptor gene expression in the virus-infected CNS in the absence of inflammation, we analyzed expression of selected chemokine and chemokine receptor genes in brains of PTI-NB rats. In these brains, we observed a sustained upregulation of CCR5 and of CX_3CR1. CX_3CR1-expressing cells were predominantly microglia. In the hippocampus, a site of neuronal cell loss in PTI-NB rats, we found strong expression of CX_3CR1, but reduced expression of the CX_3CR1 ligand, fractalkine. The possible relevance of these findings with respect to neurodegenerative processes in brains of PTI-NB rats are discussed next.

Results

Chemokine and chemokine receptor gene expression in the CNS following intracerebral BDV infection of adult rats

To examine the kinetics of chemokine receptor gene expression in the CNS following intracerebral BDV infection of adult rats, BDV-infected and sham-infected control rats were sacrificed at different times after infection, and cerebral RNA (excluding cerebelli) was analyzed by RPA (Figure 1A). We observed constitutive expression of CX_3CR1, CCR5, and very low levels of CXCR4 RNAs in cerebri of sham-infected animals. We did not detect constitutive expression of CXCR2 or CXCR3 genes in cerebri of sham infected adult rats.

In parallel with the first appearance of neurological symptoms and perivascular immune cell infiltrates in brains of BDV-infected rats about 11–13 days p.i. (Deschl *et al*, 1990; Sauder *et al*, 2000), we observed an increase of transcripts of all five chemokine receptor genes in brains of BDV-infected rats at

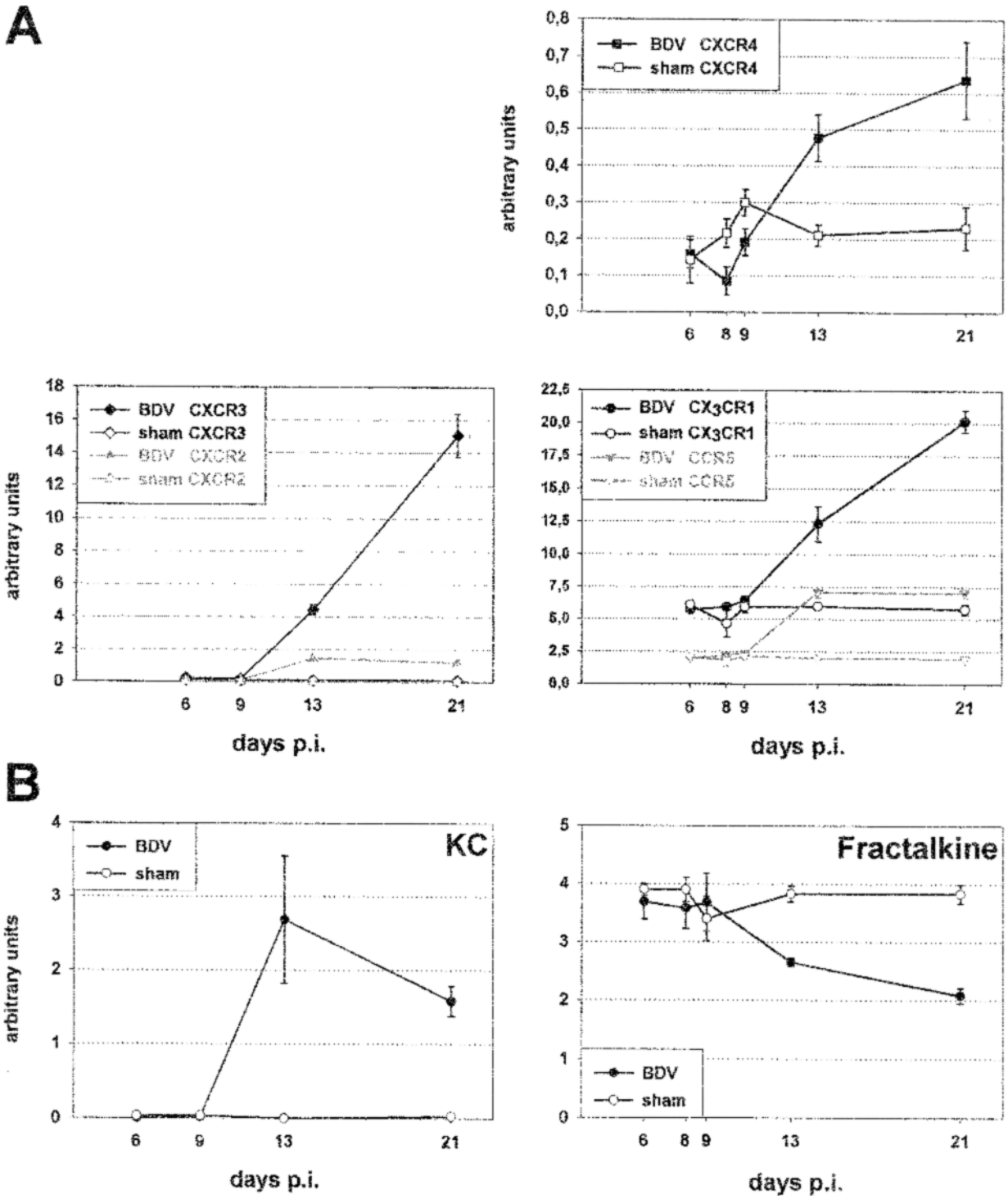

Figure 1 Chemokine and chemokine receptor gene expression in brains of adult BDV-infected rats. (**A**) Cerebral RNA samples (10 μg) (excluding cerebelli) of sham- and BDV-infected rats sacrificed at different days p.i. (3–4 animals per group and time point) were subjected to two independent RPAs using either probes for CX_3CR1, CCR5, CXCR4, and L32, or CXCR2, CXCR3, and L32, respectively. For quantitation, the dried RPA gels were exposed to phosphorimager plates, and the band intensities were determined using MacBAS software. Dots indicate mean values ± standard errors per time point p.i. after normalization against L32 expression levels (values represent arbitrary units). Expression levels of CXCR4 were low. Thus, to better illustrate the increase in CXCR4 mRNA expression in the BDV-infected brains, quantitation values for CXCR4 are depicted in a separate panel. (**B**) Cerebral RNA samples (10 μg) (excluding cerebelli) of sham- and BDV-infected rats sacrificed at different days p.i. (2–5 animals per group and time point) were subjected to two independent RPAs using either probes for KC and L32, or fractalkine and L32, respectively. See Figure 1A for description of quantitation procedure and further informations on graphs.

day 13 p.i. In parallel with the strong increase of inflammatory cells until day 21 p.i., the last time point investigated, expression levels of CX_3CR1, CXCR4, and CXCR3 genes reached maximum values at this time point. In contrast, CCR5 and CXCR2 expression levels did not further increase after day 13 p.i.

Previously, we had described upregulation of IP-10, RANTES, MIP-1β, and MCP-1 gene expression in brains of adult BDV-infected rats (Sauder *et al*, 2000). Our finding of upregulation of CX_3CR1 and CXCR2 transcripts in brains of these rats prompted us to also analyze expression of the respective ligands, fractalkine and KC/MIP-2, respectively, in these brains. RPA analysis of KC and MIP-2 gene expression in brains of BDV- and sham-infected rats sacrificed at different days p.i. revealed upregulation of KC, but not of MIP-2 gene expression at days 13 and 21 p.i. in BDV-infected brains (Figure 1B and data not shown). Consistent with the CXCR2 gene expression kinetics, KC expression levels already reached maximum values at day 13 p.i. in the infected brains. As expected, high amounts of fractalkine transcripts were found in brains of sham-infected rats (Figure 1B). Surprisingly, however, in brains of BDV-infected rats, we observed a decrease of almost 50% in the amount of fractalkine transcripts compared to control rat brains at day 21 p.i. (Figure 1B). Statistical data analysis by two-way ANOVA revealed that the overall differences between the two groups achieved statistical significance ($P = 0.0011$, $F = 13.71$).

Chemokine and chemokine receptor gene expression in brains of newborn BDV-infected rats

Besides immune cells, activated microglia might account for the observed upregulation of CCR5 and CX_3CR1 in brains of adult infected rats. To analyze whether CCR5 and CX_3CR1 expression is altered in brains of PTI-NB rats, which display strong microglial activation in the absence of immune cell infiltration, RPA was performed using RNA prepared from cerebelli, hippocampi, and frontal cortices of newborn BDV- or sham-infected rats sacrificed at different time points p.i. (Figure 2A). As expected from previous reports, constitutive expression of both genes was detectable in brains of sham infected rats at all time points analyzed (Figure 2A). In the hippocampi, frontal cortices, and cerebelli of PTI-NB rats, we measured a sustained, statistically significant twofold to threefold increased expression of the CX_3CR1 gene compared to control rats, beginning between days 27 and 33 p.i. (Figure 2A). Similarily, the amount of CCR5 transcripts in cerebelli of PTI-NB rats was found to be about twofold increased after day 21 p.i. compared to controls. CCR5 expression levels in hippocampi and frontal cortices of PTI-NB rats were only slightly increased (30–50%), beginning at days 33 p.i. or 21 p.i., respectively. Overall differences in CCR5 expression levels between the two groups over the entire time course were found to be statistically significant in cerebelli and frontal cortices, but not in hippocampi (Figure 2A).

To address the question whether the increased expression of CX_3CR1 in PTI-NB rat brains is accompanied by alterations in fractalkine gene expression, RPA was performed using RNA prepared from hippocampi and frontal cortices of PTI-NB and control rats sacrificed at different times p.i. (Figure 2B). Cerebellar fractalkine gene expression was not analyzed, since fractalkine levels were reported to be very low in the rat cerebellum (Nishiyori *et al*, 1998). Interestingly, in frontal cortices of PTI-NB rats, fractalkine mRNA levels were found to be reduced at days 21, 27, and 33 p.i. compared to control animals. We also measured reduced fractalkine gene expression in hippocampi of PTI-NB rats compared to control rats after day 14 p.i. Data analysis by two-way ANOVA revealed that the overall differences between the two groups achieved statistical significance in both brain regions [hippocampus: $P \leq 0.0001$ ($F = 59.00$); frontal cortex: $P = 0.0025$ ($F = 10.61$)]. Thus, similar to the situation in brains of adult BDV-infected rats, fractalkine expression levels were reduced in the PTI-NB rat brain.

Identification of CX_3CR1 gene-expressing cells in PTI-NB and control rat brains

To localize the cellular source of CX_3CR1 RNA in PTI-NB rat brains, *in situ* hybridization (ISH) was performed. Sections from paraffin-embedded brains prepared from PTI-NB and control rats sacrificed after different times p.i. were probed with antisense and sense CX_3CR1 probes. Two rats each were analyzed per group and time point (days 14, 21, 27, 33, and 48 p.i.) except for days 8 and 75 p.i., with only one brain each being analyzed per group. A representative autoradiograph of brain sections from each one 33-day-old PTI-NB and control rat is depicted in Figures 3A and B. Consistent with a previous report (Nishiyori *et al*, 1998), CX_3CR1-specific signals were uniformly distributed over the whole brains of sham-infected rats. In good agreement with our RPA results, elevated levels of CX_3CR1 were observed in brains of PTI-NB rats beginning around day 27 p.i. Besides an increased expression of CX_3CR1 throughout the brains, some brain regions, such as the frontal cortex and cerebellar white matter displayed higher expression levels compared to other brain regions, which was most prominent at day 33 p.i. This was also true for the dentate gyrus of the hippocampus (Figure 3B). Hybridization of brain sections with sense CX_3CR1 probes yielded only very faint background signals (data not shown).

Visualization by dark field microscopy of CX_3CR1 positive cells revealed a higher density of CX_3CR1 positive cells in brains of PTI-NB rats compared to controls (Figures 3C, D, and data not shown). In frontal cortices and hippocampi of PTI-NB rats, some cells appeared to display higher expression levels of CX_3CR1 as compared to cells in respective regions of

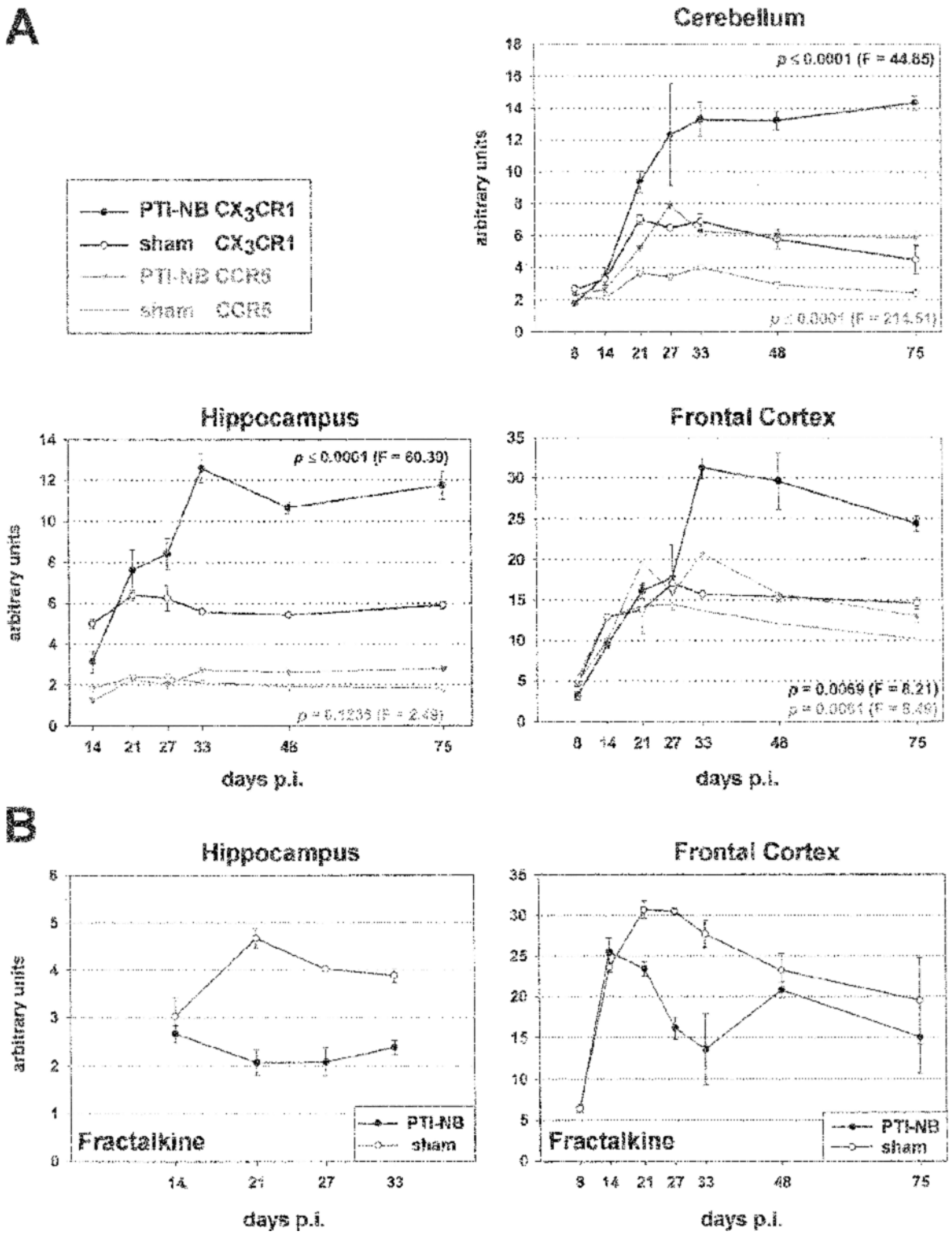

Figure 2 Chemokine and chemokine receptor gene expression in brains of PTI-NB rats. (**A**) CX_3CR1 and CCR5 gene expression in different brain regions of PTI-NB rats. Total RNA (10 μg) extracted from the cerebellum, hippocampus, and frontal cortex of neonatally sham-infected or BDV-infected (PTI-NB) rats sacrificed at different days p.i. (3–5 animals were employed per group and time point) were subjected to RPA using probes for CCR5, CX_3CR1, and L32. The large number of samples used for expression analysis over the entire time course required performance of two separate RPAs for each brain region, encompassing days 8 to 27 p.i. and days 27 to 75 p.i., respectively, in case of cerebellum and frontal cortex, or days 14 to 33 p.i. and days 33 to 75 p.i., respectively, in case of the hippocampus. Graphs each represent combined quantitation results of the two RPAs per brain region. See Figure 1A for description of quantitation procedure and further informations on graphs. To test whether overall differences in CCR5 and CX_3CR1 mRNA levels between the two groups over the entire time course achieved statistical significance, two-way ANOVA was used ($\alpha = 0.05$). Group effect *P*- and *F*-values are indicated in the respective graphs (letters in black: CX_3CR1-values; letters in grey: CCR5 values). (**B**) Reduced expression of fractalkine mRNA in hippocampi and frontal cortexes of PTI-NB rats. Total RNA (10 μg) extracted from the hippocampus and frontal cortex of neonatally sham-infected or BDV-infected (PTI-NB) rats, sacrificed at different days p.i. (3–5 animals per group and time point), were subjected to RPA using probes for fractalkine and L32. Quantitated results of the RPAs are depicted in the respective graphs. In case of frontal cortex, for technical reasons, two separate RPAs were performed encompassing days 8 to 27 p.i. or days 27 to 75 p.i. In the right panel, combined quantitation results of the two RPAs are shown. For further informations on graphs, see Figure 1A.

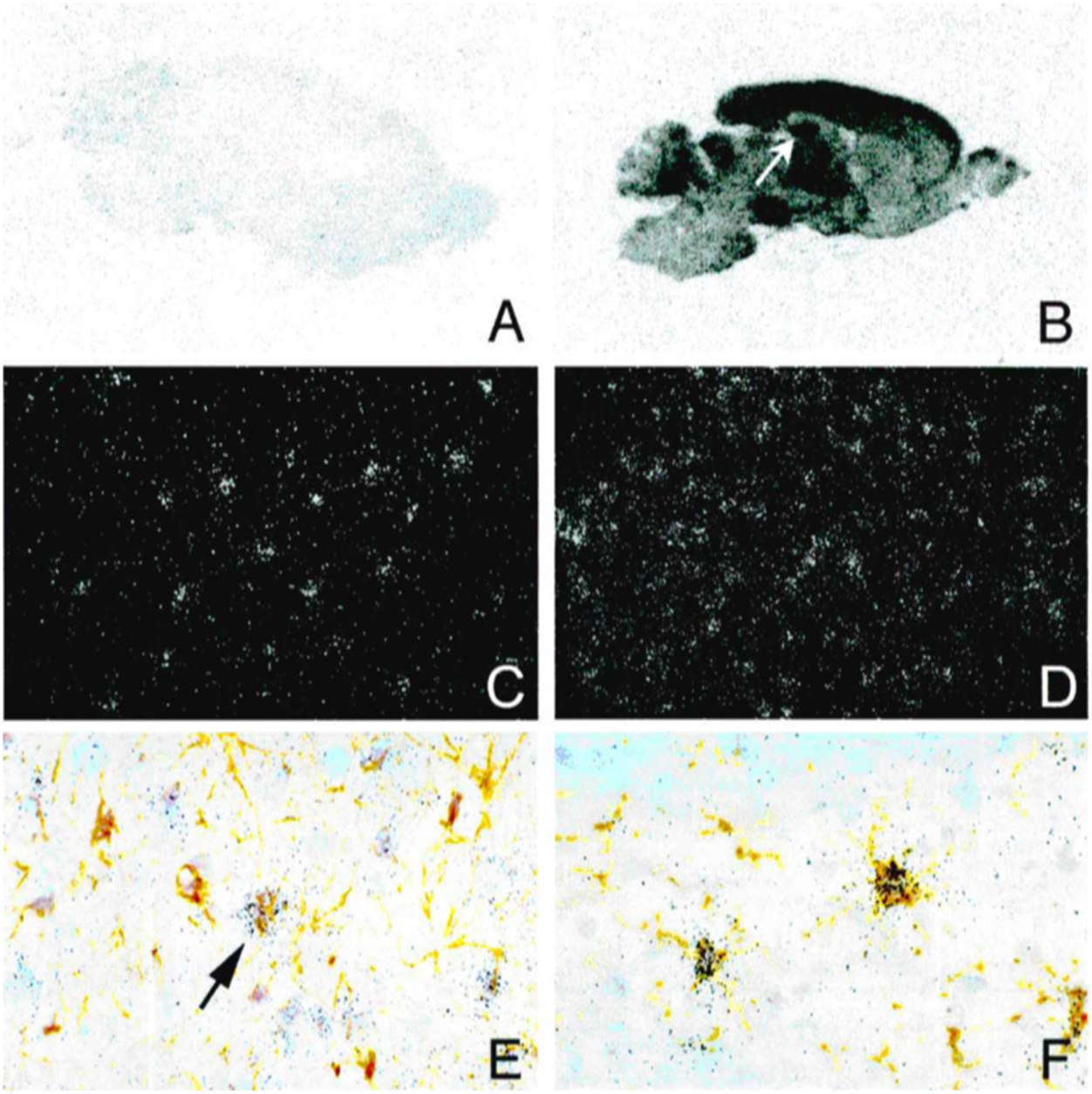

Figure 3 Visualization and determination of the cell-type of CX_3CR1 mRNA-expressing cells in brains of PTI-NB rats by ISH. Rats were sham-infected (**A**, **C**) or BDV-infected (PTI-NB; **B**, **D**, **E**, **F**) as neonates and sacrificed at 33 days p.i. Following perfusion with 4% buffered PFA, brains were removed and either subjected to paraffin embedding procedures (**A–E**) or cryoprotected in 10% sucrose/PBS and subsequently frozen (**F**). (**A–D**) Sagittal sections derived from one PTI-NB rat brain and one age-matched control rat brain each were mounted on the same slide and were probed with P^{33}-labeled antisense riboprobes specific for rat CX_3CR1 [riboprobe lengths were 606 nucleotides in case of **A** and **B**, and 1060 nucleotides in case of **C** and **D**]. In **A** and **B**, a scanned autoradiograph after 5 days' exposure to film is depicted. Pictures **C** and **D** were taken using dark field microscopy following exposure of the slide to emulsion for 5.5 weeks. Note strong ISH signals in the neocortex and dentate gyrus (marked by an arrow) of the PTI-NB rat brain (**B**). Following ISH using the 1060-nt CX_3CR1-specific riboprobe, a section from a paraffin embedded brain was subjected to IHC using a GFAP specific polyclonal rabbit serum (**E**). A section from a frozen brain was first stained with a CD11b-specific monoclonal antibody and then probed with the 1060-nt CX_3CR1-specific riboprobe (**F**). Sections depicted in **E** and **F** were counterstained with Mayer's hematoxylin. CX_3CR1-positive cells in **F** are CD11b-positive cells, thus representing microglia. A CX_3CR1-expressing GFAP-positive astrocyte is indicated by an arrow (**E**). Original magnifications in C–F each were 200×.

control animals. Thus, increased amounts of CX_3CR1 transcripts in brains of PTI-NB rats most likely are primarily due to an increase in the number of CX_3CR1 expressing cells.

To determine the cell type(s) expressing CX_3CR1 in BDV-infected brains, combined ISH and immunohistochemistry was performed using either GFAP or CD11b (Integrin alpha M-chain; clone OX-42) -specific antibodies as markers for astrocytes or microglia/macrophages, respectively. GFAP and CD11b stainings were done using paraffin- or cryosections, respectively, from brains prepared from PTI-NB and control rats sacrificed at days 14 to 75 p.i. or days 27 and 33 p.i., respectively (two rats each were used per group and time point). The vast majority of CX_3CR1-positive cells in brains of sham-infected control animals were found to be CD11b positive, displaying a morphology typical for resting (ramified) microglia (data not shown). Consistent with previous reports (Weissenböck *et al*, 2000), staining of brain sections of PTI-NB rats with mAb OX-42 revealed massive microgliosis in infected brains. Most of the CX_3CR1 expressing cells in brains of PTI-NB rats stained positive for CD11b and exhibited a morphology typical for resting and reactive (activated) microglia (Figure 3F and data not shown). We also observed some scattered CX_3CR1-positive cells that showed immunoreactivity for GFAP in PTI-NB and control brains, suggesting that few astrocytes may produce this receptor (Figure 3E and data not shown).

Discussion

This study was aimed at further elucidating the regulation of chemokine genes and their receptors in virus-infected brains of rats both in the presence and in the absence of CNS inflammation. Our previous finding that chemokine gene expression immediately preceded the onset of inflammation in brains of adult BDV-infected rats (Sauder *et al*, 2000) suggested that the specific pattern of chemokines expressed at the site of infection might determine the specific composition of immune cell infiltrates. During the acute phase of Borna disease, cerebral immune cell infiltrates predominantly consist of T cells and macrophages and to a lesser extent of NK cells. Low numbers of B cells also can be detected. Among T cells, CD4 T cells outnumber CD8 T cells (Deschl *et al*, 1990; Hatalski *et al*, 1998). This picture is considered to be typical for a Th1-type immune response. The increased cerebral expression of chemokine receptors CX_3CR1, CXCR3, CXCR4, and CCR5 in adult BDV-infected rats in the early phase of inflammation demonstrated here is in agreement with the presence of activated T cells, macrophages, NK cells, and B cells. As increased expression of CXCR3 and CCR5 in brains of infected rats correlated well with prominent expression of the respective ligands in these brains, namely IP-10 in case of CXCR3, or MIP-1β and RANTES in case of CCR5, our data lend further support to the concept that the generation of a Th1-type immune response in the acute phase of BD is primarily mediated by chemokines.

The detection of CXCR2 RNA was unexpected, since neutrophils have not been observed in BDV-infected brains. In fact, the low CXCR2 transcript levels suggest the presence of only few neutrophils. The observed low level expression of KC, the CXCR2 ligand, in infected rat brains is in line with the published finding that transgenic expression of KC in mouse oligodendrocytes resulted in cerebral infiltration of neutrophils (Tani *et al*, 1996). Whether CNS resident cells or immune cells are the source of KC expression in our model remains to be defined.

CX_3CR1 RNA levels were found to be upregulated in brains of adult BDV-infected rats. This likely can be attributed to both the influx of leukocytes into the CNS, as well as to microglia proliferation. Surprisingly, expression of fractalkine, the ligand of CX_3CR1, was reduced in brains of infected rats. In these brains, neuronal cell loss takes place during the acute phase of disease (Narayan *et al*, 1983; Bilzer and Stitz, 1994). At day 13 p.i., the amount of fractalkine transcripts was found to be reduced by about 30% compared to control rats. At this time point, however, only 4% of cortical neurons were reported to be damaged in BDV-infected rat brains (Bilzer and Stitz, 1994). Thus, it is unlikely, that the observed decrease in the amount of fractalkine mRNA merely reflects loss of fractalkine expressing neurons.

Despite increased levels of IP-10 in brains of PTI-NB rats, only a mild, transient inflammation mainly confined to the frontal cortex takes place in the brains of PTI-NB rats at about 4–5 weeks p.i. (Sauder and de la Torre, 1999; Weissenböck *et al*, 2000). This is most likely due to the assumed tolerance of T cells to BDV antigens in these animals and the fact that IP-10 only is able to induce activated, but not naïve T cells to cross the blood–brain barrier. Accordingly, only very low levels of CXCR3 mRNA can be detected in brains of PTI-NB rats (M. Rauer and C. Sauder, unpublished observations). Although expression levels of CXCR2 and CXCR4 and its ligands KC and SDF-1 in brains of these rats were only marginally altered compared to controls (M. Rauer and C. Sauder, unpublished results), a significant upregulation of CX_3CR1 and CCR5 was observed. Our data indicate that CX_3CR1 upregulation is primarily due to an increase in the number of CX_3CR1-positive cells, namely microglia/macrophages, and it is in agreement with the previous observation of massive microglial activation and proliferation in the PTI-NB rat brain (Sauder and de la Torre, 1999; Weissenböck *et al*, 2000). CCR5 was also described to be expressed on microglia (Jiang *et al*, 1998). Thus, increased CCR5 levels are likely due to an increased number of CCR5-positive microglia as well. Since monocyte attractant RANTES is upregulated in brains of PTI-NB rats (Sauder *et al*, 2000), it is possible that

macrophages, which are indistinguishable from activated microglia, are recruited into the CNS by RANTES, thereby contributing to increased CCR5 and CX_3CR1 expression. Increased expression of CX_3CR1 has also been found in spinal cords of rats following induction of EAE (Jiang *et al*, 1998), as well as in the rat facial motor nucleus following facial motor nerve axotomoy. In the latter case, increased CX_3CR1 expression paralleled the increase in microglial numbers after axotomy (Harrison *et al*, 1998). Taken together, these findings point to an increased expression of CX_3CR1 in the CNS upon various insults, including virus infection.

Similar to brains of adult BDV-infected rats, we found reduced levels of fractalkine mRNA in hippocampi and frontal cortices of PTI-NB rats. Because hippocampal fractalkine mRNA already was decreased at 3 weeks p.i. when apoptosis in the hippocampus still is minimal (Hornig *et al*, 1999; Zocher *et al*, 2000), it seems unlikely that reduced levels of fractalkine are merely due to loss of fractalkine expressing granule neurons. However, loss of these neurons likely contributes to decreased fractalkine levels at later time points p.i.. Intriguingly, fractalkine mRNA levels in the frontal cortex were only transiently reduced 3–5 weeks p.i., coinciding with transient inflammation in this brain region. Together, our findings in adult as well as newborn BDV-infected rats suggest that the regulation of fractalkine mRNA expression is influenced by the presence of an inflammatory reaction. While the exact function of fractalkine and its receptor in the CNS still needs to be defined, a role of fractalkine in signaling from neurons to microglia has been suggested (Harrison *et al*, 1998; Nishiyori *et al*, 1998). This view was supported by the finding that fractalkine induces microglial cell migration and activation *in vitro* (Maciejewski-Lenoir *et al*, 1999).

Only limited information is available concerning the regulation of fractalkine transcription and translation. Using an *in vitro* model of glutamate-triggered neurotoxicity, Chapman *et al* (2000) showed that fractalkine mRNA expression was unaffected, whereas the amount of soluble secreted fractalkine protein was increased. Similarly, in the rat model of facial motor nerve axotomy, increased amounts of lower molecular mass forms of fractalkine protein were detected despite decreased levels of neuronally expressed fractalkine mRNA (Harrison *et al*, 1998). Whether reduced fractalkine mRNA levels in the BDV-infected brain translate in reduced protein levels, or rather an increase in the amount of soluble fractalkine is unknown. The latter assumption would fit well with our observation of strong CX_3CR1 signal intensities in the dentate gyrus (DG) of PTI-NB rats around day 33 p.i. when neuronal apoptosis and microglial activation in this region are most intense (Hornig *et al*, 1999; Zocher *et al*, 2000; Weissenböck *et al*, 2000). Because microglia are capable of secreting both neurotrophic and neurotoxic molecules, the question whether microglial activation is beneficial or harmful to neurons is still a matter of debate (Streit *et al*, 1999). Likewise, the role of microglia activation in brains of PTI-NB rats, especially with regard to DG granule cell death still is unclear. Attraction of microglia by soluble fractalkine via CX_3CR1 therefore might indirectly promote or counteract neuronal cell death.

More recent studies indicate that fractalkine may exhibit anti-inflammatory functions. Accordingly, fractalkine was shown to control and suppress certain aspects of microglial activation, such as TNF-α secretion following LPS treatment (Zujovic *et al*, 2000, 2001). Expression of several proinflammatory, potentially neurotoxic cytokines, most likely secreted by activated microglia, has been found in brains of PTI-NB rats (see Carbone *et al*, 2001, for a review). Therefore, an involvement of these cytokines in selective neuronal cell death in these brains cannot be excluded. It is tempting to speculate that soluble fractalkine (despite reduced mRNA levels) is produced in brains of these rats in order to counteract or weaken the otherwise harmful effects of these cytokines. Alternatively, assuming that the observed reduced expression of fractalkine mRNA in brains of PTI-NB rats translates into reduced fractalkine protein levels, it is conceivable that an efficient anti-inflammatory and possibly antitoxic function of fractalkine towards activated microglia is impaired. Reduced fractalkine levels therefore might lead to an imbalance between above mentioned beneficial and harmful functions of microglia and thereby might indirectly contribute to neuronal damage.

In conclusion, our findings are in line with the idea that members of the chemokine/chemokine-receptor system are involved in pathogenic events that lead to neurodegeneration. The exact role of chemokine receptors and their ligands in promoting or counteracting neurodegenerative processes awaits further investigation. Targeting the function of specific chemokines or their receptors might turn out to be a novel approach to alter the course of neurological disease.

Materials and methods

Virus stocks

The BDV stocks (kindly provided by O. Planz and L. Stitz, Tübingen) used for infection of newborn or adult rats were the fourth brain passages (BDVRp4) in newborn or adult Lewis rats, respectively, of rat BDV (Staeheli *et al*, 2000). Infectious titers and preparation of virus stocks are described in Sauder *et al* (2000) and Zocher *et al* (2000).

Infection of rats

Pregnant Lewis rats (Charles River, Sulzfeld, Germany) were monitored twice daily. Newborns were inoculated intracerebrally within 16 h after

birth with 30 μl of BDVRp4 (ca 1×10^4 focus forming units (f.f.u.)), or—for sham infections—with 30 μl of a brain homogenate (10% (wt/vol) suspension in PBS) derived from an adult rat. None of the newborn infected rats showed symptoms of BD. Beginning at day 21 p.i., PTI-NB rats exhibited reduced body weight as expected (Carbone *et al*, 1991). Four- to 6-week-old male Lewis rats were anesthetized using methoxyflurane (Methofane, Janssen-Cilag, Neuss, Germany), and 50 μl of BDVRp4 (1.5×10^4 f.f.u.), diluted in Dulbecco's modified Eagle medium containing 2% fetal bovine serum, or 50 μl of pure diluent (sham infection) were injected into the left brain hemisphere.

Collection and preparation of tissues for histological analysis and ISH

Rats were euthanatized with CO_2 at different time points p.i. and immediately perfused transcardially with icecold sterile PBS followed by icecold 4% buffered paraformaldehyde (PFA). Brains were removed, postfixed in 4% buffered PFA for 3–4 h at RT, and cryoprotected by immersion in 20% sucrose (w/v)/PBS overnight at 4°C. Brains then were embedded in GSV-1 tissue-embedding compound (SLEE Technik, Mainz, Germany), snap-frozen in liquid nitrogen-cooled isopentane, and stored at −70°C. Sagittal cryostat sections (10 μm) were mounted onto polylysine-coated slides and stored at −20°C. PFA-fixed, paraffin-embedded rat brains were used from previous studies (Zocher *et al*, 2000; days 8–48 p.i.; Sauder *et al*, 2000; day 75 p.i.). Sagittal sections (8 μm) were cut, dried overnight at 37°C, and stored at 4°C.

Preparation of RNA

At various time points p.i., rats were euthanatized with CO_2. The cerebelli, hippocampi, and frontal cortices of neonatally infected rats, and the cerebri (excluding the cerebelli) of rats infected as adults, were quickly removed, immediately frozen in liquid nitrogen and stored at −70°C until RNA was prepared. Total RNA was isolated using TRIZOL (GibcoBRL, Karlsruhe, Germany) as described previously (Zocher *et al*, 2000). To remove possible DNA contaminations of RNA preparations, which were employed to examine presence of CXCR2 and CXCR3 mRNA transcripts by RPA, RNA samples were treated with DNase I (Ambion, Austin, TX) followed by phenol extraction and ethanol precipitation.

Plasmid constructs

To generate probes encoding partial sequences of chemokine and chemokine receptor genes, total RNA was prepared from whole brains of adult uninfected or BDV-infected rats, as well as from cerebelli of newborn sham- or BDV-infected rats. RNAs were reversely transcribed using oligo(dT). The resulting cDNAs were employed to amplify by PCR fragments of rat chemokine genes KC, MIP-2, and fractalkine, and rat chemokine receptor genes CXCR2, CXCR3, CXCR4, CX_3CR1, and CCR5. PCR was done using rat or mouse (in case of CXCR3) specific primers flanked by *Hind*III or *Bam*HI (sense primers) and *Eco*RI (antisense primer) sites or vice versa, which allowed subcloning of the amplified DNA fragments into vectors pGEM-3Z or pGEM-4Z. The rat fractalkine gene was first amplified by PCR using mouse fractalkine-specific primers as used in Pan *et al* (1997). The obtained fragment was subloned into vector pCR2.1 (Invitrogen), sequenced, and used to amplify by PCR a 610-bp subfragment using a rat fractalkine sense primer (flanked by a *Bam*HI site) and the same antisense primer as used before. A 377-bp *Bam*HI-*Pst*I fragment, derived from the 610-bp subfragment was subcloned into pGEM-4Z. The identity of the respective subcloned fragments was verified by sequence analysis. All sequences were as published before (Table 1), except for one to three single point

Table 1 Specifications of plasmids used for RPA and ISH studies[a]

Gene	*Subcloned sequence (position in gene)*	*Protected length*[b]	*Vector*	*Polymerase and restriction enzyme used for antisense (sense) transcript*	*GenBank accession no.*	*Reference*
CCR5	810–1025	216	pGEM-3Z	T7/*Hind*III	U77350	Jiang *et al*, 1998
CXCR2	254–594	341	pGEM-3Z	T7/*Hind*III	U70988	Dunstan *et al*, 1996
CXCR3	395–833[b]	254	pGEM-4Z	SP6/*Hind*III	AF223642	Wang *et al*, 2000
CXCR4	421–681	261	pGEM-3Z	T7/*Hind*III	U90610	Jiang *et al*, 1998
CX_3CR1	246–536 (RPA)	291	pGEM-3Z	T7/*Hind*III		
	410–1015 (ISH)	606	pGEM-3Z	T7/*Hind*III (SP6/*Eco*RI)	U04808	Harrison *et al*, 1994
	59–1118 (ISH)	1060	pGEM-3Z	T7/*Hind*III (SP6/*Eco*RI)		
Fractalkine	589–965	377	pGEM-4Z	T7/*Eco*RI	AF030358	Harrison *et al*, 1998
KC	64–429	366	pGEM-3Z	T7/*Hind*III	M86536	Huang *et al*, 1992
MIP-2	57–300	244	pGEM-3Z	T7/*Hind*III	X65647	Driscoll *et al*, 1995

[a]For information on the plasmid encoding L32 sequences, see Sauder *et al* (2000).
[b]The region of the CXCR3 sequence protected in RPA encompasses nucleotides 580–833 ISH: *in situ* hybridization.

mutations in some of the probes. See Sauder *et al* (2000) for description of the plasmid containing a partial sequence of the housekeeping gene that codes for the ribosomal protein L32. See Table 1 for sequence positions and lengths of the subcloned gene fragments, restriction enzymes, and polymerases used for generating antisense and sense RNA probes for RPA and ISH.

Ribonuclease protection assay

For synthesis of the radiolabeled antisense riboprobes, plasmids encoding segments of the respective genes (Table 1) were linearized, purified, and adjusted to a concentration of 25 ng/μl. To simultaneously detect CXCR4, CCR5, CX_3CR1, and L32 transcripts, radiolabeled riboprobes of the respective plasmids first were synthesized in two independent parallel reactions containing both CXCR4 mixed with CCR5, as well as CX_3CR1 mixed with L32. Reactions each were carried out in a volume of 10 μl containing transcription buffer (1×, Promega, Madison, WI, USA), 100 nmoles DTT, 20 nmoles each of rGTP, rATP, rCTP; 1 μl of template mixture; 70–80 μCi [α-^{32}P] UTP (800 Ci/mmol); 25–50 pmoles of rUTP, RNase inhibitor (28 U, Pharmacia), and T7 RNA polymerase (10 U, Promega). After 1 h incubation at 37°C, samples were heated for 5 min at 93°C, cooled immediately on ice for 2 min, and the template DNAs eliminated by treatment with DNase I (2 U, Ambion) for 30 min at 37°C. After extraction with phenol-chloroform, probes were precipitated with ethanol in the presence of NH_4Ac and 10 μg of glycogen (Roche Molecular Biochemicals, Mannheim, Germany) as carrier, dried, and dissolved (2.5–3 × 10^5 cpm/μl) in hybridization buffer (40 mM PIPES (pH 6.4), 0.4 M NaCl, 1 mM EDTA, and 80% formamide). Target RNA (10 μg) was dried under vacuum and resuspended in 15 μl of hybridization buffer containing each 1 μl of the CCR5/CXCR4 and CX_3CR1/L32 riboprobe mixtures. All other riboprobes were individually synthesized. Generation of radiolabeled riboprobes was done as described previously using 60–80 μCi [α-^{32}P] UTP (800 Ci/mmol) and 0–100 pmoles rUTP. Both transcripts of KC and MIP-2 as well as CXCR2 and CXCR3 were analyzed simultaneously together with L32. Transcripts of fractalkine were detected individually together with L32. Hybridizations each were done using 2–3 × 10^5 cpm of the fractalkine, KC, and MIP-2 riboprobes, and 1.5–2 × 10^5 cpm of the L32 riboprobe per target RNA sample. For simultaneous detection of CXCR2, CXCR3, and L32 transcripts, synthesized riboprobes were gel-purified before hybridization as previously described (Sauder and de la Torre, 1999). 1–2 × 10^5 cpm each of these purified riboprobes were precipitated together with 10 μg of target RNA and resuspended in 15 μl of hybridization buffer. All RPAs were further carried out as described previously (Zocher *et al*, 2000), except for RPAs analyzing CXCR2 and CXCR3. RNase digestion mixtures in these RPAs contained only 1/5 of the amounts of RNase A and RNase T1 used in the other RPAs.

Combined ISH—immunohistochemistry (IHC)

ISH procedure was performed as described previously (Sauder *et al*, 2000). Two different CX_3CR1 probes (Table 1) were tested by ISH. Following completion of the riboprobe synthesis reaction and following 30 min incubation at 37°C in the presence of DNase I (0.7 U, Ambion), the longer CX_3CR1 probe (1064 b) was subjected to alkaline hydrolysis. Briefly, 45 μl of ETS buffer (10 mM Tris pH 7.4; 10 mM EDTA; 0.2% SDS), 1.67 μl 5 M NaCl, and 1 μl 1 M DTT were added to the sample and incubated on ice for 5 min. Alkaline hydrolysis was initiated by addition of 10 μl 2 M NaOH. After incubation on ice for 40 min, the reaction was stopped by addition of 20 μl 2 M HEPES. The probe then was precipitated and further processed in the same manner as the shorter probe according to the described procedure. Both antisense riboprobes yielded comparable signal intensities when hybridized to rat brain sections derived from the same brains and when sections were exposed to Ultra Vision G film (Sterling, Newark, Del.) for 5 days. To simultaneously visualize astrocytes and CX_3CR1-expressing cells in paraffin sections, ISH for CX_3CR1 was followed by IHC using a rabbit polyclonal serum against GFAP (DAKO, Hamburg, Germany) as described previously (Sauder *et al*, 2000). To simultaneously detect CX_3CR1-expressing cells and microglia/macrophages in cryosections of PFA-treated, sucrose-cyroprotected rat brains, IHC was performed prior to ISH. Cryosections (10 μm) were thawed, air-dried, and immersed twice for 2 min in PBS. Sections were blocked at RT for 30 min in blocking solution (PBS, 5% horse serum (Vector Laboratories, Burlingame, CA), 0.2% Triton X-100, 5000 U/ml heparin (porcine; Calbiochem)). After washing thrice in PBS (5 min each), sections were incubated for 1 h at RT with mouse monoclonal antibody OX-42 (anti-rat CD11b; Serotec, Oxford, England) at a dilution of 1:100 in blocking solution.

After washing three times in PBS (5 min each), sections were incubated at RT for 30 min with a biotinylated secondary horse antimouse antibody (rat absorbed; Vector Laboratories), diluted 1:200 in blocking solution. Bound antibody was detected with an avidin-biotin-peroxidase kit (ABC-Elite; Vector Laboratories) and diaminobenzidine as a substrate. For ISH, sections were postfixed in PBS-4% formaldehyde, washed twice in PBS, and incubated for 7 min in a solution of pronase protease (*Streptomyces griseus*; Calbiochem) at a final concentration of 24 U/ml in 50 mM Tris-HCl, pH 7.5; 5 mM EDTA. Pronase activity was stopped by incubation of sections for 30 s in 0.2% glycine/PBS solution followed by washing twice in PBS, 3 min each. Sections then were acetylated in 0.25% acetic anhydride in PBS for 10 min. After another 5-min fixation in PBS-4%

formaldehyde, slides were dehydrated in graded alcohols and dried. Sense and antisense CX_3CR1 probe synthesis, hybridization, and further processing of sections was done as described (Sauder *et al*, 2000). After final dehydration of sections in graded alcohols, slides were air dried and exposed for 4–5 days to Ultra Vision G film. Slides then were dipped in Kodak NTB-2 emulsion, dried, and stored in the dark for 5 weeks. Subsequently, slides were developed, counterstained with Mayer's hematoxylin, dehydrated in graded alcohols, mounted, and examined by dark and bright field microscopy.

Statistical analysis

Logarithmic two-way analysis of variance (ANOVA) with groups (BDV and sham; 3–5 animals each per time point analyzed) and days p.i. as independent factors was employed to calculate group, day, and group × day effects for mRNA expression of chemokine and chemokine receptor genes ($\alpha = 0.05$). Group effect values are indicated in the respective experiments. Day and group × day effect values are not shown. Statistical analysis was done with procedure GLM of SAS 6.12 software package (Cary, NC, USA).

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