Identification of chemoattractive factors involved in the migration of bone marrow-derived mesenchymal stem cells to brain lesions caused by prion

Running title: Chemotactic factors for MSCs to prion-specific lesions

Chang-Hyun Song,[1†] Osamu Honmou,[2] Hidefumi Furuoka,[3] and Motohiro Horiuchi[1]*

[1]Laboratory of Veterinary Hygiene, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan; [2]Departments of Neural Repair and Therapeutics, Sapporo Medical University, South-1st, West-16th, Chuo-ku, Sapporo 060-8543, Japan; [3]Department of Pathobiological Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro 080-8555, Japan

[†]Present address: Department of Neurology, Emory University School of Medicine, Woodruff Memorial Building, 101 Woodruff Circle, Atlanta, GA 30322, USA

[*]Correspondence to: Motohiro Horiuchi, DVM, Ph.D.
Laboratory of Veterinary Hygiene,
Graduate School of Veterinary Medicine,
Hokkaido University,
Kita 18, Nishi 9, Kita-ku,
Sapporo 060-0818, Japan
Phone/Fax: +81-11-706-5293
e-mail: horiuchi@vetmed.hokudai.ac.jp

ABSTRACT

Bone marrow-derived mesenchymal stem cells (MSCs) have been reported to migrate to brain lesions of neurodegenerative diseases; however, the precise mechanisms by which MSCs migrate remain to be elucidated. In this study, we carried out *in vitro* migration assay to investigate chemoattractive factors for MSCs in the brains of prion-infected mice. The migration of immortalized human MSCs (hMSCs) was reduced by their pre-treatment with antibodies against the chemokine receptors**,** CCR3, CCR5, CXCR3 and CXCR4 and by pre-treatment of brain extracts of prion-infected mice with antibodies against the corresponding ligands, suggesting the involvement of these receptors and their ligands in the migration of hMSCs. In agreement with the results of an *in vitro* migration assay, hMSCs in the corpus callosum, which are considered to be migrating from the transplanted area towards brain lesions of prion-infected mice, expressed CCR3, CCR5, CXCR3 and CXCR4. The combined *in vitro* and *in vivo* analyses suggest that CCR3, CCR5, CXCR3 and CXCR4 and their corresponding ligands are involved in the migration of hMSCs to the brain lesions caused by prion propagation. Additionally, hMSCs that had migrated to the right hippocampus of prion-infected mice expressed CCR1, CX3CR1 and CXCR4, implying the involvement of these chemokine receptors in hMSC functions after chemotactic migration. Further elucidation of the mechanisms that underlie the migration of MSCs may provide useful information regarding application of MSCs to the treatment of prion diseases.

INTRODUCTION

Prion diseases are fatal neurodegenerative disorders in humans and animals that are characterized by the accumulation of a disease-specific isoform of the prion protein (PrP^{Sc}), astrocytosis, microglial activation, spongiosis and neuronal cell death in the central nervous system (CNS). Although the etiology of the diseases is not clear, conversion of the normal prion protein to PrP^{Sc} plays a key role in the neuropathological changes (44). Therefore, compounds that inhibit PrP^{Sc} formation are considered as therapeutic candidates of the diseases and many compounds have been reported to inhibit PrP^{Sc} formation in cell cultures and cell-free systems [reviewed in (56)]. However, only a few of these inhibitors, such as amphotericin B and its derivative (13), pentosan polysulfate (14), porphyrin derivatives (27) certain amyloidophilic compounds (25) and FK506 (37) have been reported to prolong the survival of prion-infected mice even when administered in the middle-late stage of infection, but still before clinical onset. We recently reported that intraventricular infusion of anti-PrP antibodies (50) slowed down the progression of the disease even when initiated just after clinical onset. However, in addition to inhibition of PrP^{Sc} formation, protection of neurons or restoration of degenerated neurons is thought to be important for functional recovery.

Bone marrow-derived mesenchymal stem cells (MSCs) differentiate into cells of mesodermal origin such as adipocytes, osteoblasts and endothelial and muscle cells (41, 43). In addition, MSCs are known to trans-differentiate into neuronal and glial cells. MSCs have been shown to migrate to damaged neuronal tissues and to alleviate the deficits in experimental animal models of cerebral ischemia (10), spinal cord injury (20), Parkinson's diseases (19, 33) and amyotrophic lateral sclerosis (59). MSCs also secrete various

68 neurotrophic factors that may protect neuronal tissues from degradations as well as stimulate
69 the activity of endogenous neural stem cells (38). Therefore, despite their mesodermal
70 origin, MSCs are considered to be a candidate for cell-mediated therapy for
71 neurodegenerative diseases. One of the characteristics of MSCs is their migration to brain
72 lesions of neurodegenerative diseases including prion diseases (10, 19, 39, 51). This feature
73 may be of further use for cell-mediated therapy of neurodegenerative diseases, particularly for
74 prion diseases, Multiple sclerosis and Alzheimer's disease, which have diffuse pathological
75 lesions.

76 Since many cytokines, chemokines and adhesion molecules are involved in the homing of
77 immune cells (9, 36, 53), evidence that a variety of chemokines and growth factors, as well as
78 their cognate receptors have a pivotal role in the migration of MSCs has been accumulated.
79 These factors include CXCL12 and its receptor CXCR4 [(30, 40) and reviewed in (52)],
80 CCL2 (15, 62, 66), CCL3 (62), IL-8 (48, 62), hepatocyte growth factor (16), platelet-derived
81 growth factor-AB (PDGF-AB), insulin-like growth factor-1 (IGF-1), CCL5 and CCL22 (42),
82 and integrin β1 (23). Regarding migration of MSCs to injury in the CNS, involvement of
83 CCL2 (61), CXCL12/CXCR4 and CX3CL1/CX3CR1 (24) have been reported. However,
84 knowledge of the mechanism by which MSCs migrate to pathological lesions of
85 neurodegenerative diseases is insufficient and further efforts are required to elucidate this
86 mechanism.

87 We recently reported that human MSCs (hMSCs) migrate to CNS lesions and prolong the
88 survival of mice infected with prion (51). In the present study we investigated factors that
89 are involved in the migration of hMSCs to brain lesions of prion diseases.

90

MATERIALS AND METHODS

Cell culture

Human bone marrow-derived MSCs that were immortalized with the human telomerase catalytic subunit gene (26), and that stably expressed the LacZ gene [hMSCs, (51)] were used. The hMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum (FBS) in a humidified atmosphere under normoxic (21% O_2 and 5% CO_2) or hypoxic (2% O_2 and 5% CO_2) conditions at 37 °C.

Mice and prion inoculation.

All animal experiments were carried out according to protocols approved by the Institutional Committee for Animal Experiments. Four-week-old female ICR mice were purchased from CLEA Japan Inc. (Japan), and the mice were acclimatized for a week prior to use. Brain homogenates [10% (w/w) in phosphate-buffered saline (PBS)] that were used for inoculation were prepared from brains of mice infected with the Chandler prion strain at the terminal stage of the disease and from age-matched uninfected mice. Mice were intracerebrally inoculated with 20 μl of 10% brain homogenates.

In vitro migration assay

To examine the migration of hMSCs *in vitro*, the brains of infected mice 120 days post inoculation (dpi) with the Chandler strain, or of age-matched mock-infected mice, were homogenized to 20% (w/w) in DMEM. The homogenates were centrifuged at 10,000 × *g* for

10 min at 4 °C, and the resulting supernatants were passed through a 0.22-μm pore size filter. Aliquots of the brain extracts were stored at –80 °C until use. Migration of hMSCs to the brain extracts was assessed using a QCM^{TM} chemotaxis cell migration assay kit (Chemicon, Temecula, CA). The hMSCs that were starved for a day in serum-free medium were harvested, and 300 μl of cell suspension (5×10^4 cells) was added to the insert well. The lower chambers were supplied with serum-free DMEM containing 1% brain extract. Twenty-four hours after incubation, hMSCs on the poly-carbonate membrane (pore size 8.0 μm) were stained with the cell stain solution provided in the kit. Non-migratory cells that stayed on the upper side of the poly-carbonate membrane were removed using a cotton swab. The migrated hMSCs, which had passed through the pores and clung to the under-side of the membrane, were counted using the NIH Image J program. Three random high-magnification (100×) light microscopic images were captured using the Olympus BX-51 microscope, and were used for cell counting.

To examine the involvement of receptors expressed on hMSCs in their migration, hMSCs were pre-incubated with antibodies against specific receptors for 30 min before adding the insert wells. To examine the involvement of chemokines and growth factors in migration, 1% brain homogenates were incubated with antibodies against specific chemokines and growth factors for 30 min at 4 °C prior to adding to the insert wells. Antibodies against the following proteins were purchased from the indicated companies: PDGF-αβ receptor (PDGF-αβR, ab34074); CCR2 (ab1668); CCR4 (ab1669); CX3CR1 (ab7201); CXCR3 (clone 2Ar1) and CCL3 (ab10381), all from Abcam (Cambridge, MA)**;** IGF-1 receptor (IGF-1R; clone 33255); CXCR4 (clone 12G5); IGF-1 (AF791); CCL2 (clone 123602); CCL4 (AF-451-NA); CCL5 (AF-478); CCL7 (AF-456-NA); CCL17 (AF-529); CCL24 (clone

106521); CX3CL1 (clone 126315); CXCL10 (AF-466-NA); CXCL12 (clone 79014) and CXCL13 (AF-470), all from R&D systems (Minneapolis, MN); CCR1 (clone #141-2) and CCR3 (clone 444-11), both from MRL; CCR5 (Sigma Chemical Co., clone 45523.111); PDGF-AB (Millipore, Billerica MA, #06-127). All antibodies were used at a concentration of 10 μg/ml. Migration assays were performed as three independent experiments (each experiment was carried out in triplicate).

Transplantation of hMSCs

The hMSCs were transplanted into the thalamus as described previously (51). Briefly, after anesthesia, mouse scalps were incised. The mice were then placed onto a stereotaxic apparatus (Narishige, Japan), and burr holes were drilled to accommodate stereotaxic placement into the thalamus (Bregma - caudal 2.0 mm, lateral 2.1 mm and depth 3.2 mm). The hMSCs (1×10^5 cells in 2 μl DMEM) were transplanted over a period of 15 min using a Hamilton syringe with a 31-gauge needle.

Flow cytometric analysis

The hMSCs were treated with 1mM EDTA and dispersed in 0.5% FBS in PBS (FBS-PBS) by pipetting. The cells were then incubated with primary mouse antibodies against IGF-1R, CCR1, CCR3, CCR5, CXCR3 and CXCR4; primary goat antibodies against PDGF-αβR, CCR2 and CCR4, and with a primary rabbit antibody against CX3CR1 (all 1:200 dilution) in 0.5% FBS-PBS for 30 min on ice. The primary antibodies were omitted for negative controls. The cells were washed three times with 0.5% FBS-PBS and incubated with anti-mouse Alexa Fluor 546, anti-goat Alexa Fluor 555 or anti-rabbit Alexa Fluor 555

(Molecular Probes, Eugene, OR) (1:1000 dilution) for 30 min on ice. After washing, the cells were stained with 5 μg/ml of propidium iodide (Molecular Probes) in PBS for 5 min, and analyzed using an EPICS XL-ADC flow cytometer (Beckman Coulter, Miami, FL).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was obtained from hMSCs or from mouse brains using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). First-strand cDNA was synthesized from 2.5 μg of the total RNA using a First-strand synthesis kit (Amersham Biosciences, UK) according to the manufacturer's instructions. Quantitative PCR was carried out using a TaqMan assay. The amplification reaction mixtures contained template cDNA, 1× pre-designed TaqMan Gene Expression Assays and 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in a final reaction volume of 20 μl. The following TaqMan gene expression assays were purchased from Applied Biosystems; human genes for PDGF-αβR (assay ID; Hs00182163), CCR3 (Hs00266213), CCR4 (Hs99999919), CCR5 (Hs99999149), CX3CR1 (Hs00365842), CXCR3 (Hs01847760) and CXCR4 (Hs00607978), and mouse genes for CCL3 (Mm00441259), CCL4 (Mm00443111), CCL5 (Mm01302427), CCL7 (Mm00443113), CCL17 (Mm01244826), CX3CL1 (Mm00436454), CXCL10 (Mm00445235) and CXCL12 (Mm00445553). The human RNase P gene or the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified as an internal marker using human RNase P control reagents (Applied Biosystems, 4316844) or TaqMan rodent GAPDH control reagents (Applied Biosystems, 4308313). TaqMan assays were carried out using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The amplification profiles were analyzed using the threshold cycle (CT) relative quantification method, and were normalized

to the expression of the human RNase P control gene or the mouse GAPDH gene, which were used as human and mouse reference genes as described previously (58).

Immunofluorescence assay (IFA)

IFA was performed on hMSCs cultured in an 8-well chamber slide (Nalge Nunc, Naperville, IL) or on cryosections (5 μm thick) of mouse brains after transplantation of hMSCs. The cells or sections were fixed with cold methanol for 20 min at -20 °C, and were treated with PBS containing 0.1% polyoxyethylene (20) sorbitan monolaurate (Tween-20) (PBST) for 10 min. After blocking with 5% FBS in PBST for 30 min, the cells or sections were incubated for 1 h with primary antibodies for the receptors described above using a 1:500 dilution. After washing with PBST, they were then incubated with a 1:2000 dilution of anti-mouse Alexa Fluor 546, anti-goat Alexa Fluor 555 or anti-rabbit Alexa Fluor 555 for 1 h at room temperature. To investigate the co-localization of hMSCs with their receptors, the sections were incubated for 90 min with a mouse anti-β-gal antibody (Cat No. Z3783, Promega, Madison, WI) conjugated with Alexa Fluor 488 (51). After washing with PBST, samples were then mounted with Vectashield containing 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Samples were observed under a Nikon C1 laser confocal microscope.

RESULTS

Migration of hMSCs to the brain extracts

206 Hypoxic pre-conditioning of MSCs is reported to enhance their migratory activity (2, 22).
207 To determine the effects of oxygen conditions on the migration of hMSCs to brain
208 homogenates of mock- or prion-infected mice, hMSCs that were cultured under normoxic or
209 hypoxic conditions were analyzed using an *in vitro* migration assay. The hMSCs that were
210 pre-cultured under hypoxic conditions migrated to brain extracts of prion-infected mice more
211 efficiently than those pre-cultured under normoxic conditions (Fig. 1A). Quantitative
212 analysis revealed that twice as many hMSCs migrated to the brain extracts of prion-infected
213 mice than to those of mock-infected mice following normoxic pre-conditioning, while 2.7
214 times more hMSCs migrated to the brain extracts of prion-infected mice following hypoxic
215 pre-conditioning (Fig. 1B). No differences were observed in the migration of hMSCs to the
216 brain extracts of mock-infected mice between normoxic and hypoxic pre-conditioning. We
217 therefore used hMSCs that were pre-cultured under hypoxic conditions for all subsequent
218 experiments to facilitate discrimination between subtle differences in cell migration. These
219 hMSCs showed a time-dependent increase in migration for up to 24 h (Fig. 1C), and their
220 migration increased in a brain-extract concentration-dependent manner (Fig. 1D). Migration
221 of hMSCs to 0.01% and 0.001% brain homogenates of prion-infected mice was observed,
222 whereas only migration to 1% brain homogenates, but not to lower percentages of brain
223 extracts of mock-infected mice was observed.
224
225 **Chemotactic factors involved in the migration of hMSCs**
226 The chemokine CXCL12 and its receptor, CXCR4, are known to be involved in the
227 chemotactic migration of MSCs to brain lesions of neurodegenerative diseases (24, 63).
228 However, as MSCs express a variety of receptors for growth factors, chemokines and

229 cytokines that are associated with cell migration (8, 52), it was anticipated that other
230 cytokines, chemokines and growth factors also play a role in the migration of MSCs. We
231 therefore selected 10 receptors for chemokines and growth factors, and 13 of their ligands, as
232 indicated in Table 1, and analyzed their involvement in hMSC migration using an *in vitro*
233 migration assay (Fig. 2). These factors were chosen with reference to cell surface expression
234 of those receptors on MSCs in previous studies (50), and to the pathway and networks of
235 receptors and their ligands obtained using Ingenuity Pathway Analysis 5.0 (Ingenuity System
236 Inc., Redwood City, CA). The migration of hMSCs to the brain extracts of prion-infected
237 mice was significantly decreased when hMSCs were pre-treated with antibodies against
238 CCR3, CCR4, CCR5, CXCR3 and CXCR4, compared with the migration of non-treated
239 hMSCs ($p < 0.05$, Dunnett's tests) (Fig. 2A). When antibodies against the ligands of these
240 receptors were tested (Table 1), the migration of hMSCs was significantly decreased by
241 treatment of the brain extracts with antibodies against the ligands for CCR3 (CCL5, CCL7
242 and CCL24), CCR5 (CCL3, CCL4 and CCL5), CXCR3 (CXCL10) and CXCR4 (CXCL12)
243 ($p < 0.05$) (Fig. 2B). Neither the antibody against CX3CR1, nor that against its
244 corresponding ligand, CX3CL1, decreased the migration of hMSCs. In addition, the
245 antibody against CCR4 decreased hMSC migration, whereas the antibody against its ligand,
246 CCL17, did not. When the involvement of growth factors and their receptors in migration
247 was analyzed, antibodies against the PDGF-αβR or the IGF-1R did not decrease migration,
248 whereas antibodies against their respective ligands, PDGF-AB and IGF-1, did decrease
249 migration. In addition, an antibody against CCR2 or its ligand CCL2 did not decrease
250 migration, but an antibody against CCL7, another ligand for CCR2, did decrease migration.
251 Taking into account of biological relationship between the receptors and ligands that were

analyzed here, the migration assays suggested that at least CCR3, CCR5, CXCR3 and CXCR4, and their ligands were involved in the migration of hMSCs to the brain extracts of prion-infected mice.

The expression of chemokine and chemokine receptor genes.

We next examined the expression of chemokine genes in the brains of prion-infected mice and that of chemokine receptor genes in hMSCs treated with the brain extracts of prion-infected mice. For the latter analysis, hMSCs were incubated with DMEM containing 1% brain extracts of prion- or mock-infected mice. Twenty-four hours after incubation, total RNA was recovered and gene expression was analyzed using qRT-PCR. The expression of CCR3, CCR4, CCR5 and CXCR4 genes was elevated 2- to 9-fold in hMSCs treated with the brain extracts of prion-infected mice compared to their expressions in hMSCs that were treated with the brain extracts of mock-infected mice (Fig. 3A). In contrast, the expression of PDGF-αβR, CX3CR1 and CXCR3 genes was not up-regulated by stimulation of the hMSCs with brain extracts from prion-infected mice.

The expression of chemokine genes in the brains of prion-infected mice or of age-matched control mice was also analyzed at 120 dpi. The expression of CCL3, CCL4, CCL5 and CXCL10 genes was up-regulated by 10-fold (CXCL10, CCL5) to nearly 50-fold (CCL3, CCL4) in the brains of prion-infected mice (Fig. 3B). In addition, the mRNA levels of CCL7 and CCL17 (a ligand for CCR4) were moderately increased (about 3 to 6-fold) in the brains of prion-infected mice. In contrast, expression of the CX3CL1 and CXCL12 genes was not up-regulated.

Expression of chemokine receptors on hMSCs.

Flowcytometric analysis and IFA were carried out to confirm the expression of chemokine and growth factor receptors on hMSCs. The hMSCs treated with brain extracts of mock-infected mice expressed PDGF-αβR, CCR3, CCR4, CXCR4 and CXCR3 on the cell surface. Of these receptors, expression of CCR3, CCR4 and CXCR4 was increased by stimulation with brain extracts of prion-infected mice, whereas no increase in expression of PDGF-αβR and CXCR3 was observed. Although it is difficult to distinguish signals on the plasma membrane from those in the cytoplasm using IFA, a similar tendency was observed in IFA; CCR3, CCR4 and CXCR4 fluorescent signals appeared to be more intense in cells treated with brain extracts of prion-infected mice than in cells treated with brain extracts of mock-infected mice. In contrast to the expression of these receptors, hMSCs expressed a trace level of CX3CR1, and CCR5 was not detectable on the cell surface. However, the expression of CCR5 was specifically induced in response to brain extracts of prion-infected mice (Fig. 4A). The prion-specific induction of CCR5 expression was also confirmed by IFA (Fig. 4B).

Differential expression of chemokine receptors on hMSCs after transplantation into the brains of prion-infected mice

It is known that MSCs transplanted into the left hippocampus or thalamus migrate to the contralateral (right) hippocampus through the corpus callosum (3, 19, 51). Therefore, we hypothesized that receptors expressed on hMSCs in the corpus callosum are possibly involved in the migration of hMSCs to neuropathological lesions. We therefore transplanted hMSCs into the left thalamus of prion- or mock-infected mice at 120 dpi, and analyzed the expression

of growth factor/chemokine receptors on hMSCs. Two days after transplantation of hMSCs into the left thalamus of prion-infected mice, hMSCs in the transplanted region expressed CCR1, CCR3, CCR4, CCR5, CX3CR1, CXCR3 and CXCR4 (Fig. 5A) as well as PDGF-αβR, IGF-1R and CCR2 (data not shown). However, the hMSCs that were transplanted into the mock-infected mice showed weak expression of these receptors except for CXCR3, suggesting that the expression of these chemokine receptors was increased by stimulation with factors produced in the brains of prion-infected mice. At 2 days post-transplantation, hMSCs in the corpus callosum of prion-infected mice still strongly expressed CCR3, CCR5, CXCR3 and CXCR4, but expression of CCR1, CX3CR1 and CCR4 appeared to be lower than that on hMSCs in the transplanted area (Fig. 5B). The expression of chemokine receptors on hMSCs in the contralateral hippocampus also differed from that on hMSCs in the transplanted region and in the corpus callosum; hMSCs preferentially expressed only CCR1, CX3CR1 and CXCR4 in the contralateral hippocampus at one week post-transplantation (Fig. 5B). These data suggest that CCR1, CX3CR1 and CXCR4 may be associated with specific activities of hMSCs after their migration to the target lesions.

DISCUSSION

MSCs are known to migrate to neuropathological lesions of neurodegenerative diseases (8, 54). The migrated MSCs can contribute to the functional recovery of damaged nervous tissues by secretion of various trophic factors (12), neuronal differentiation or cell fusion (1, 29), and stimulation of the proliferation and differentiation of endogenous neural stem cells

(38). To date, transplantation of MSCs has been reported to ameliorate the symptoms not only of the experimental animal models of neurodegenerative diseases (10, 19, 20, 33, 59), but also of human patients with multiple system atrophy (32), amyotrophic lateral sclerosis (35) or stroke (4). The involvement of CXCL12 and its cognate receptor CXCR4 in MSC migration to injured tissues is well-established (17) in spite of some exceptions (23). *In vitro* migration assays reported to date have identified growth factors and chemokines that are possibly involved in MSC migration (42, 49, 60, 61). However, the mechanisms of MSCs migration are expected to differ with tissue microenvironments induced by diseases. Targeting of MSCs to neuropathological lesions is essential for functional recovery, therefore, an understanding of the mechanisms that underlie the migration of MSCs to lesions in the CNS could contribute to the development of MSC-mediated cell therapy by facilitating site-specific migration of MSCs. The involvement of CCL2, CXCL12/CXCR4 and CXCL1/CX3CR1 in the migration of MSCs to brain lesions has been reported (24, 62, 63). However, the mechanisms that underlie migration of MSCs to neuropathological lesions are largely unknown.

We recently showed that hMSCs can migrate to neuropathological lesions induced by prion propagation (51). Since brain extracts of prion-infected mice were considered to contain chemoattractive factors (5, 46, 57, 65), we analyzed factors that induce hMSC migration by blocking experiments using antibodies against receptors for growth factors and chemokines and their ligands. To increase the accuracy of the interpretation of *in vitro* migration assays, we defined a ligand/receptor interaction as chemoattractive if antibodies against both the ligand and its cognate receptor reduced the migration of hMSCs. Based on this criterion, we expected that CCR3, CCR5, CXCR3 and CXCR4, and their ligands are possibly involved in

344 the migration of hMSCs to the brain extracts of prion-infected mice (Fig. 2). The
345 involvement of CXCL12/CXCR4 signaling in hMSCs migration is consistent with findings in
346 hypoglossal nerve injury (24), ischemic (63) and glioma (11) models. Although an effect of
347 CCR3, CCR5 or CXCR3 on MSC migration in brain injury has not been reported, CXCR3
348 and CCR5 are known to modulate resident microglial migration to brain lesions (6, 45). In
349 prion diseases, impairment of microglial migration, associated with the increased
350 accumulation of PrP^{Sc} but prolongation of survival, has been reported in CXCR3 gene
351 deficient mice infected with prions (47). Microglial recruitment in retina after intraocluar
352 injection of homogenates from prion-infected neuroblastoma cells was inhibited by CCR5
353 antagonist, suggesting the involvement of CCR5 in microglial response to prion infection (34),
354 although ablation of CCR5 gene did not influence the incubation period after prion infection
355 (55). Since MSCs are able to migrate to brain lesions, the mechanisms by which they do so
356 are expected to show some similarity with the mechanisms that underlie microglial migration.
357 CCL2 has been reported to mediate MSC migration to ischemic brain lesions (62). However,
358 neither an anti-CCL2 antibody, nor an antibody against its receptor, CCR2, reduced hMSC
359 migration to brain extracts of prion-infected mice, implying that CCL2/CCR2 interaction may
360 not mediate the migration of MSCs to prion-specific brain lesions. PDGF-AB and IGF-1
361 have been reported to be strong chemoattractants for MSC migration *in vitro* (42). In the
362 present study, antibodies against these two growth factors reduced hMSC migration, but
363 antibodies against their receptors did not inhibit hMSC migration despite the surface
364 expression of these receptors on hMSCs (Fig. 4). Because of the complexity of the brain
365 extracts and the limitations of blocking experiments, the lack of inhibition by these antibodies
366 against those receptors does not necessarily mean that these ligands/receptor interactions have

367 no functional relevance. Further detailed analysis will define additional factors that possibly
368 mediate the migration of MSCs in response to damage of nervous tissue caused by prion
369 propagation.

370 Regarding the expression of chemokine genes, the gene expression of CCR5 ligands
371 (CCL3, CCL4 and CCL5), CCR3 ligands (CCL5 and CCL7) and the ligand for CXCR3
372 (CXCL10) was up-regulated. The up-regulation of CXCL10 gene in the CNS following
373 viral infection has been reported to attract T lymphocytes bearing CXCR3 into the CNS (64),
374 suggesting that MSCs share some mechanism of trafficking with lymphocyte trafficking.
375 The hMSCs transplanted into the brains of mock-infected mice do not migrate, and the degree
376 of hMSC migration in the brains of prion-infected mice correlates with the progression of
377 neuropathological lesions (51). These results indicate that constitutive expression levels of
378 chemokines and/or growth factors in the brain do not, but increased levels of those factors do,
379 induce hMSC migration. Therefore, although CXCR4 and its ligand CXCL12 have been
380 reported to play an important role in migration of MSCs in the CNS (24, 63), the lack of
381 up-regulation of CXCL12 gene expression in prion-infected mice suggests that
382 CXCL12/CXCR4 interaction does not have an important role in the initial event on attraction
383 of hMSCs. Indeed, cell migration is dependent on a multitude of signals. Therefore,
384 cytokines, chemokines and growth factors, whose expression is up-regulated in the brains of
385 prion-infected mice, e.g., the CXCL10, CCL3-5 and CCL7 that we analyzed in this study, as
386 well as other factors reported previously (5, 57, 65), will stimulate hMSCs to initiate
387 migration.

388 The corpus callosum is known to be one of the sites through which MSCs transplanted into
389 one hemisphere migrate to the contralateral hemisphere (3, 19). We recently reported that

390 hMSCs transplanted into the left thalamus of prion-infected mice were detected in the corpus
391 callosum, the contralateral hippocampus and thalamus 2 days after transplantation (51). This
392 result suggests that the hMSCs that were detected in the corpus callosum were migrating to
393 the brain lesions in the contralateral hemisphere. Based on this idea, we therefore analyzed
394 the expression of chemokine receptors on hMSCs that were transplanted into prion-infected
395 mice. Interestingly, in agreement with the interpretation of the *in vitro* migration assays,
396 hMSCs in the corpus callosum clearly expressed CCR3, CCR5, CXCR3 and CXCR4 2 days
397 after transplantation (Fig. 5B), suggesting the involvement of these chemokine receptors in
398 the migration of hMSCs *in vivo*. In contrast to hMSCs in the corpus callosum, hMSCs that
399 had migrated to the contralateral hippocampus showed reduced expression of CCR3, CCR5
400 and CXCR3, whereas strong expression of CXCR4 was still observed. These results suggest
401 that CXCR4 plays a role not only in migration, but also in regulating hMSC activity following
402 chemotactic migration. A role in the regulation of hMSC activity following chemotactic
403 migration may also apply to the expression of CCR1 and CX3CR1; the weak expression of
404 CCR1 and CX3CR1 on hMSCs in the corpus callosum (Fig. 5B) appears to be consistent with
405 the results of the *in vitro* migration assay (Fig. 2). However, in contrast, these receptors
406 were clearly expressed on hMSCs in the transplanted thalamus at 2 days post-transplantation
407 and in the contralateral hippocampus of prion-infected mice a week after transplantation,
408 implying that CCR1 and CX3CR1 may play a role in regulating MSC activity after migration
409 to the targeted site.

410 Although neuroprotective functions of MSCs that have migrated to brain lesions are not
411 fully understood, the temporal and spatial differences in the expression of chemokine
412 receptors on hMSCs transplanted into the brains of prion-infected mice that we observed in

413 this study is intriguing, *i.e.*, hMSCs that are considered to be in the process of migration in the
414 corpus callosum expressed CCR3, CCR5 and CXCR3, whereas hMSCs that had migrated to
415 the target site showed reduced expression of these receptors but elevated expression of CCR1
416 and CX3CR1. The stimulation of CX3CR1 by its ligand CX3CL1 plays a role in
417 modulating the release of pro-inflammatory cytokines from microglia (7, 21) and in
418 producing neuroprotective substances (31). Therefore, expression of CX3CR1 on MSCs
419 that had migrated to the contralateral hippocampus might be an indicator of functional
420 alteration of hMSCs, *i.e.*, alteration from active migration towards the target site to exhibition
421 of neuroprotective potential. The hMSCs that had migrated to the contralateral hippocampus
422 still expressed CCR1, CX3CR1 and CXCR4 at 3 weeks post-transplantation (data not shown).
423 We have previously shown that hMSCs transplanted into prion-infected mice efficiently
424 produced various neurotrophic factors at 1 to 3 weeks post-transplantation (51). It is thus
425 conceivable that signaling via these receptors may facilitate alteration of the phenotype of
426 hMSCs to that of a neuroprotective phenotype following chemotactic migration. The
427 interaction between endogenous nucleotide and purinergic receptors on microglia is known to
428 regulate the activation state of microglia. Interaction of ATP with the $P2Y_{12}$ receptor induces
429 microglial chemotaxis to local CNS injury. However, a decrease in expression of the $P2Y_{12}$
430 receptor accompanies morphological change of microglia from a ramified to an amoeboid
431 state (18). In contrast, up-regulation of the expression of $P2Y_6$ receptors by neuronal
432 damage and interaction of UDP released from damaged neurons with the $P2Y_6$ receptor
433 triggers phagocytic activity of microglia (28). It is therefore of interest to investigate
434 whether a temporal and spatial change in the expression profile of chemokine receptors,
435 similar to the control of microglial activity by endogenous nucleotides and purinergic

receptors, accompanies alteration in the activation states of MSCs.

In this study, we showed that, in addition to CXCR4, the chemokine receptors CCR3, CCR5 and CXCR3 are involved in the migration of hMSCs to brain lesions caused by prion infection. The fact that the results of the *in vitro* migration assay are consistent with the expression of chemokine receptors on hMSCs transplanted into the brains of prion-infected mice provides strong evidence for the involvement of these chemokine receptors in the migration of hMSCs. Although factors that regulate the migration of hMSCs in the CNS may vary with diseases and with the microenvironment of the lesions, our results provide an insight into the mechanism of MSC migration towards neuropathological lesions. In addition, our results also show that comparison of MSC receptor expression in migrating MSCs with that of MSCs that had homed to the targeted site provides a clue to identification of factors that facilitate the homing of MSCs. Further investigation of the host cells that produce relevant ligands for those receptors in response to the progression of neuropathological lesions, the temporal order of receptor expression on MSCs, and of the mechanisms that regulate the activity of MSCs, may facilitate the application of MCSs to prion diseases.

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463

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682

683

684 **FIGURE LEGENDS**

685

686 **Figure 1.** Chemotactic migration of hMSCs to brain extracts of mock- or prion-infected
687 mice. The migration of hMSCs to brain extracts of mock- or prion-infected mice that were
688 prepared at 120 dpi was analyzed using a QCM^{TM} 96-well cell migration assay kit. (A)
689 hMSCs migrated to the underside of the membrane of the insert well. The hMSCs that were
690 pre-cultured under normoxic or hypoxic conditions (Normoxic or Hypoxic) were added to the
691 insert well, and the lower chambers were supplied with serum-free DMEM containing 1%
692 brain extracts of mock- or prion-infected mice (Mock or Prion). Twenty-four hours after
693 incubation, hMSCs that had passed through the pores of the membrane and clung to its

694 underside were stained (upper panel). Stained cells with a size > 200 μm^2 were selected
695 (lower panel) and were counted using the Image NIH J program. Bar = 100 μm. (B)
696 Quantification of migrated hMSCs. The migration of hMSCs, assayed as described in (A)
697 was quantified. Three random areas (3.84×10^5 μm^2/area) of the underside of the membrane
698 were photographed and the number of hMSCs was counted. The graphs show cell counts
699 per 3.84×10^5 μm^2 (mean and SD, n = 3). Black and white bars indicate the number of
700 hMSC that migrated to 1% brain extracts of mock- and prion-infected mice, respectively.
701 (C) Time-dependent increase in hMSC migration. The migration assay was performed for 4,
702 15 and 24 h. (D) Dose-dependency of hMSC migration. The migration of hMSCs to
703 various doses (0.001, 0.01, 0.1 and 1%) of the brain extracts was analyzed. The graph shows
704 cell counts per 3.84×10^5 μm^2 at the underside of the membrane (mean and SD, n = 3).
705
706 **Figure 2.** Screening of chemotactic factors involved in the migration of hMSCs. The
707 migration of hMSCs to 1% brain extracts of prion-infected mice was assessed after
708 pre-treatment of hMSCs with antibodies against receptors for chemokines or growth factors
709 (A), or after pre-treatment of brain extracts with antibodies against growth factors or
710 chemokines (B). Migration assays, in which antibody pre-treatments of hMSCs and brain
711 extracts were omitted, were assigned as control (Cont). The graphs show relative numbers
712 of hMSCs that migrated to brain extracts of prion-infected mice compared to numbers of
713 hMSCs that migrated to brain extracts in control experiments. Means and SD from three
714 independent assays (each assay was carried out in triplicate) are shown. Asterisks: $p < 0.05$
715 (Dunnett's *post hoc* test).
716

717 **Figure 3.** Expression of chemokine receptor genes in hMSCs and chemokine genes in the
718 brains of prion-infected mice. (A) Expression of chemokine and growth factor receptor
719 genes in hMSCs stimulated with brain extracts of prion-infected mice. The hMSCs were
720 incubated for a day in DMEM containing 1% brain extracts of prion-infected mice prepared at
721 120 dpi or of age-matched mock-infected mice. Expression of the mRNA of the indicated
722 genes was then analyzed using qRT-PCR. The graph shows the fold increase in gene
723 expression in hMSCs incubated with brain extracts of prion-infected mice compared to
724 hMSCs incubated with brain extracts of mock-infected mice. The results of two independent
725 experiments are shown. (B) Expression of chemokine genes in the brains of prion-infected
726 mice. The graph shows fold increase in gene expression in the brains of prion-infected mice
727 at 120 dpi compared to the brains of age-matched mock-infected mice. Mean and SD (n = 3)
728 of the fold increase are shown.
729
730 **Figure 4**. Expression of chemokine receptors on hMSCs. (A) Flow cytometric analysis.
731 The expression of chemokine receptors on the cell surface was examined after incubation of
732 the cells with brain extracts of mock- (black) or prion- (red) infected mice prepared at 120 dpi.
733 The grey histograms indicate the negative control (omitted primary antibodies). (B) IFA.
734 The hMSCs that were incubated with brain extracts of mock- or prion-infected mice (Mock or
735 Prion) for 24 h in 8-chamber slides were stained with antibodies against chemokine receptors
736 (red), and were counterstained with DAPI (blue). Bar = 20 μm.
737
738 **Figure 5.** Expression of chemokine receptors in hMSCs transplanted into the brains of
739 mock- or prion-infected mice. The hMSCs (1×10^5 cells) were transplanted into the left

thalamus of mock- or prion-infected mice at 120 dpi and cryosections of mouse brains were prepared at 2 days or a week post-transplantation. Sections were double stained with anti-β-gal antibodies conjugated with Alexa fluor 488 (green) for staining of hMSCs and with antibodies against chemokine receptors (red). Nuclei were counterstained with DAPI (blue). (A) Expression of chemokine receptors in hMSCs at the transplanted area. Images of the left thalamus (transplanted side) of mock- and prion-infected mice were taken at 2 days post-transplantation. (B) Expression of chemokine receptors in hMSCs in the corpus callosum and the right hippocampus. Images of the corpus callosum and the right hippocampus (contralateral side) of prion-infected mice were taken at 2 days and one week post-transplantation, respectively. Bar = 20 μm.

750 Table 1. Growth factor and chemokine receptors, and their corresponding ligands[a]

Receptors	Corresponding ligands
HGFR[b]	HGF
IGF-1R[c]	IGF-1
PDGF-αβR[d]	PDGF-AB
CCR1	CCL3, CCL5, CCL7, CCL13
CCR2	CCL2, CCL7, CCL8, CCL13
CCR3	CCL5, CCL7, CCL8, CCL11, CCL24
CCR4	CCL17, CCL22
CCR5	CCL3, CCL4, CCL5, CCL8, CCL11
CX3CR1	CX3CL1
CXCR3	CXCL9, CXCL10, CXCL11
CXCR4	CXCL12
CXCR5	CXCL13

751 [a] The table was referred from Spaeth *et al* (52).

752 [b] HGFR, hepatocyte growth factor receptor

753 [c] IGFR, insulin-like growth factor receptor

754 [d] PDGF-αβR, platelet-derived growth factor-αβ receptor

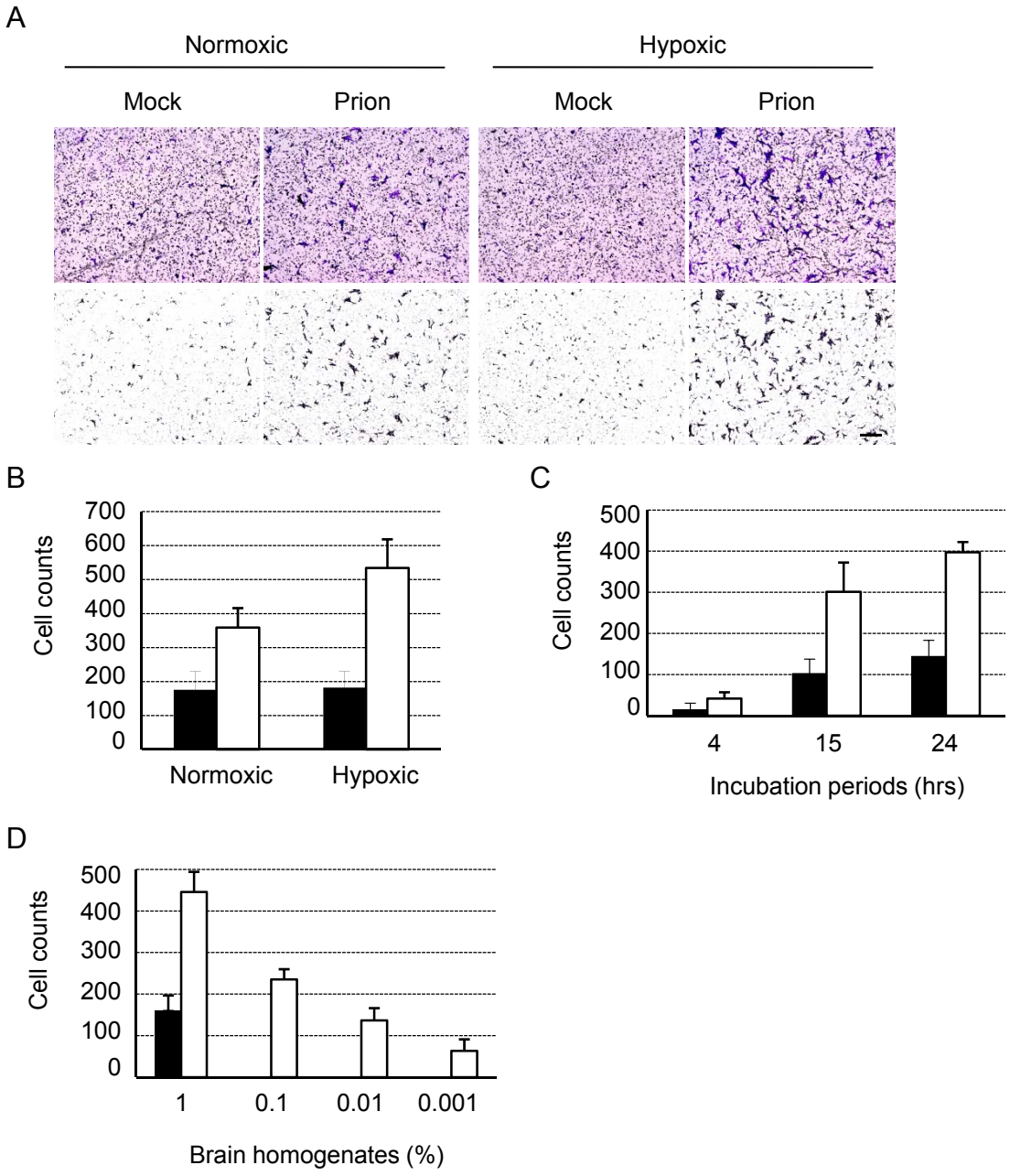
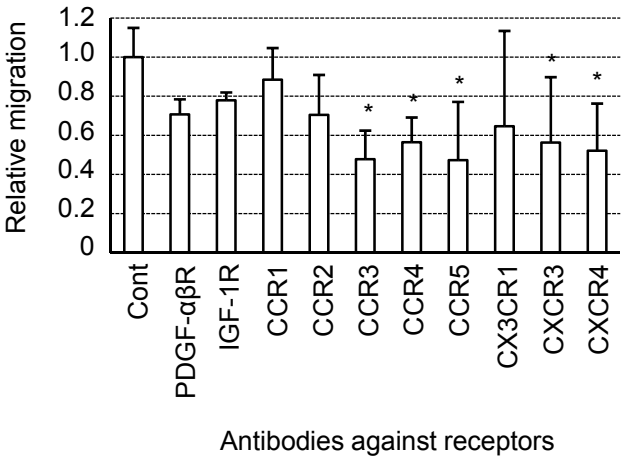


Fig. 1 Song *et al*.

A



B

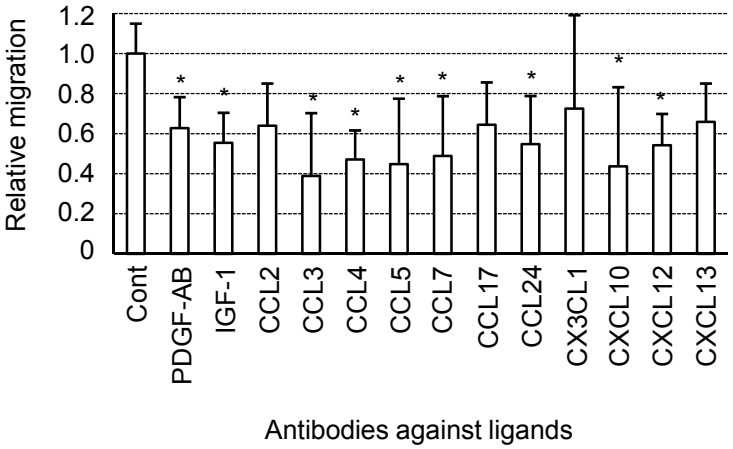


Fig. 2 Song *et al*.

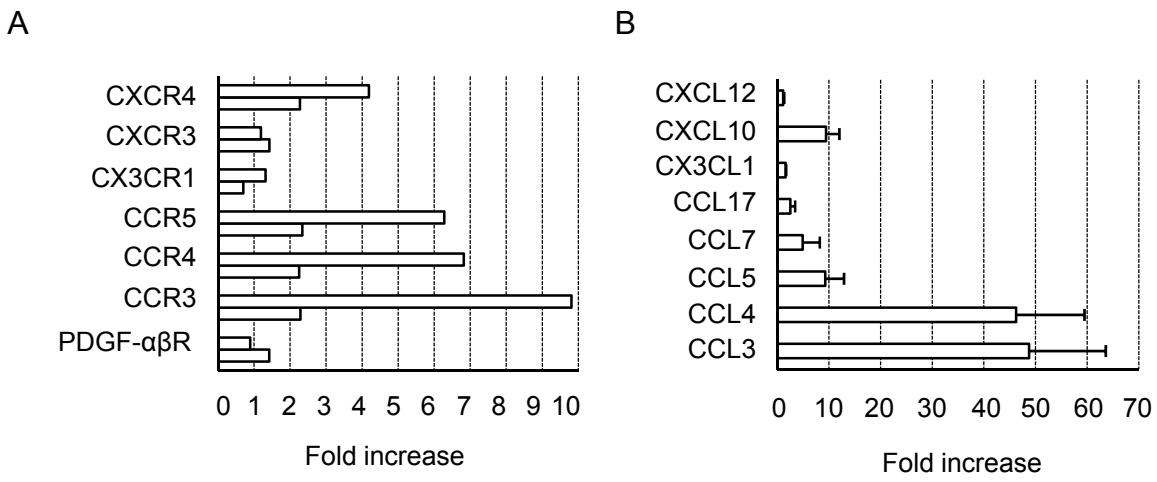


Fig. 3 Song *et al*.

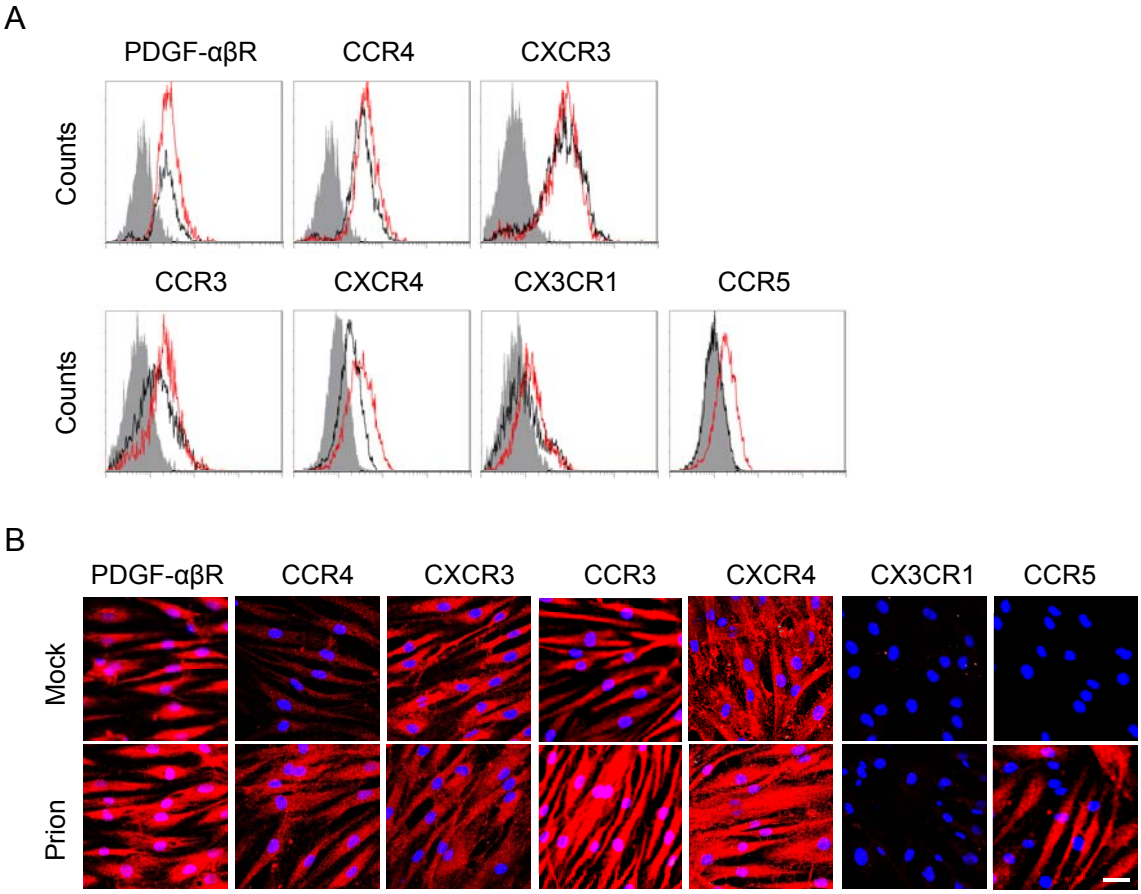


Fig. 4 Song *et al*.

A

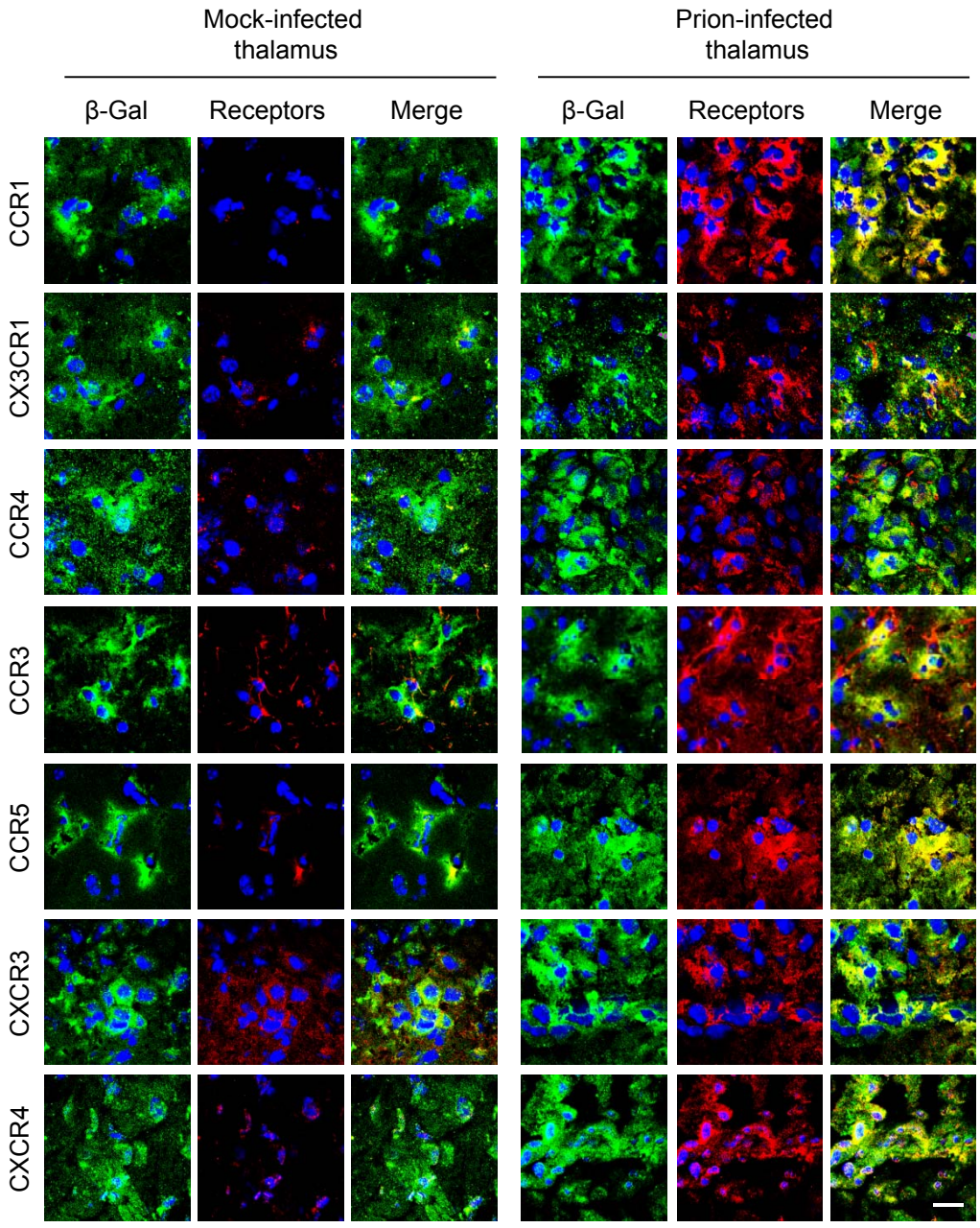


Fig. 5 Song *et al*.

To be continued

B

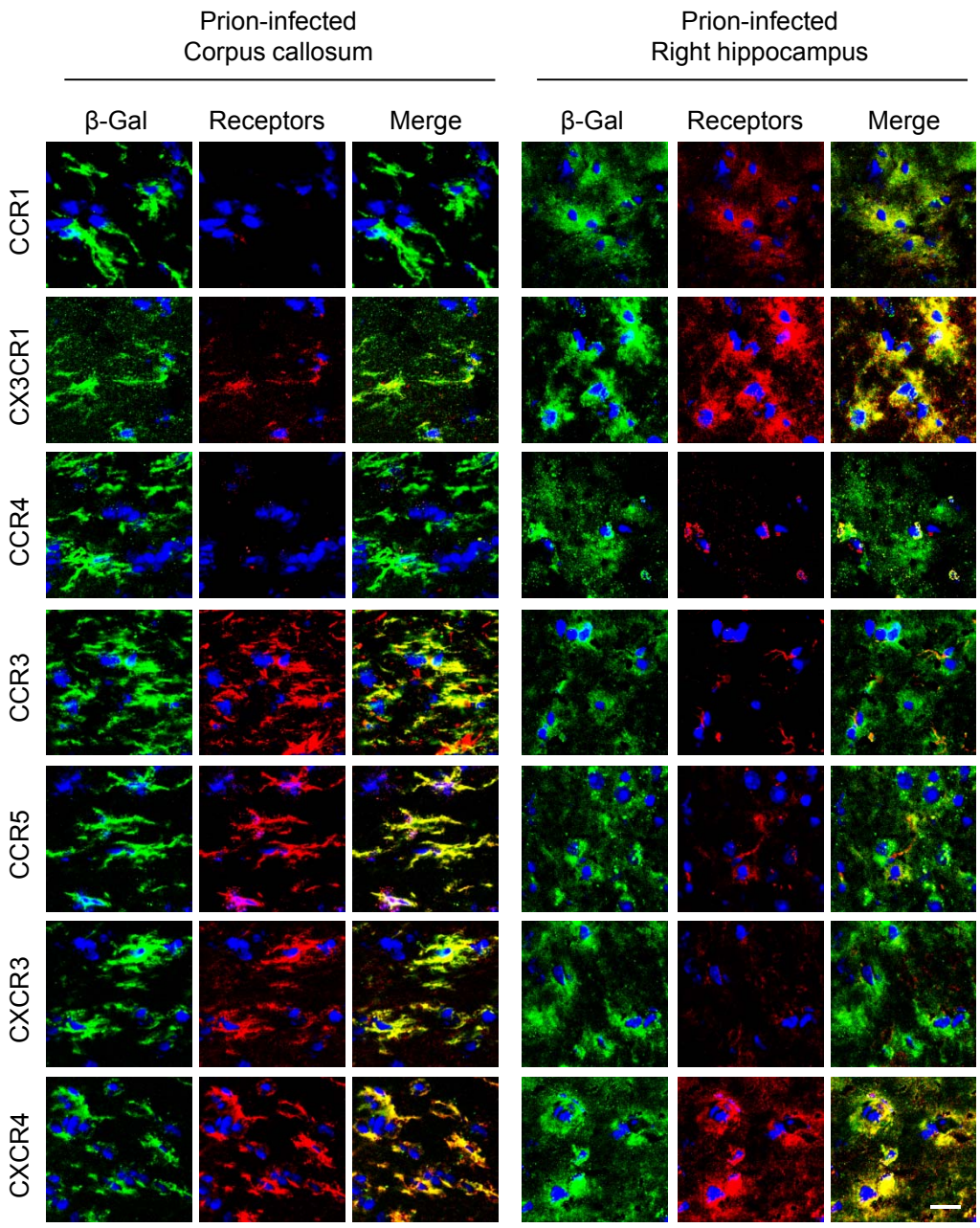


Fig. 5 Song *et al*.