

Rapid Transport of CCL11 across the Blood-Brain Barrier: Regional Variation and Importance of Blood Cells

Michelle A. Erickson, Yoichi Morofuji, Joshua B. Owen, and William A. Banks

University of Pennsylvania, Department of Pathology, School of Dental Medicine, Philadelphia, Pennsylvania (M.A.E.); Geriatric Research Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle, Washington (M.A.E., Y.M., J.B.O., W.A.B.); Department of Medicine, Division of Gerontology and Geriatric Medicine, University of Washington School of Medicine, Seattle, Washington (M.A.E., Y.M., J.B.O., W.A.B.); Department of Chemistry, University of Kentucky, Lexington, Kentucky (J.B.O.); and Department of Neurosurgery, University of Nagasaki, Nagasaki, Japan (Y.M.)

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ABSTRACT

Increased blood levels of the eotaxin chemokine C-C motif ligand 11 (CCL11) in aging were recently shown to negatively regulate adult hippocampal neurogenesis. How circulating CCL11 could affect the central nervous system (CNS) is not clear, but one possibility is that it can cross the blood-brain barrier (BBB). Here, we show that CCL11 undergoes bidirectional transport across the BBB. Transport of CCL11 from blood into whole brain (influx) showed biphasic kinetics, with a slow phase preceding a rapid phase of uptake. We found that the slow phase was explained by binding of CCL11 to cellular components in blood, whereas the rapid uptake phase was mediated by direct interactions with the BBB. CCL11, even at high doses, did not cause BBB disruption. All brain regions except striatum showed a delayed rapid-uptake

phase. Striatum had only an early rapid-uptake phase, which was the fastest of any brain region. We also observed a slow but saturable transport system for CCL11 from brain to blood. C-C motif ligand 3 (CCR3), an important receptor for CCL11, did not facilitate CCL11 transport across the BBB, although high concentrations of a CCR3 inhibitor increased brain uptake without causing BBB disruption. Our results indicate that CCL11 in the circulation can access many regions of the brain outside of the neurogenic niche via transport across the BBB. This suggests that blood-borne CCL11 may have important physiologic functions in the CNS and implicates the BBB as an important regulator of physiologic versus pathologic effects of this chemokine.

Introduction

The eotaxin chemokine C-C motif ligand 11 (CCL11) is an eosinophil chemoattractant that acts selectively through its receptor: C-C motif receptor 3 (CCR3) (Kitaura et al., 1996; Ponath et al., 1996). CCL11 has been implicated in a number of diseases that involve allergic eosinophilia, such as asthma, atopic dermatitis, and inflammatory conditions of the bowel (Rankin et al., 2000). Elevations in circulating CCL11 have also been reported in a variety of nonallergic conditions, including aging (Shurin et al., 2007; Villeda et al., 2011), obesity (Vasudevan et al., 2006), psychiatric disorders (Teixeira et al., 2008; Grassi-Oliveira et al., 2012), and cannabis use (Fernandez-Egea et al., 2013). Recently, it was shown that elevated circulating CCL11 can contribute to cognitive decline in aging by directly inhibiting adult hippocampal neurogenesis (Villeda et al., 2011). The mechanism by which circulating

CCL11 affects the central nervous system (CNS) is not characterized, but these findings raise the possibility that CCL11 somehow accesses brain tissue. One possibility that has been suggested is that CCL11 specifically enters neurogenic niches through areas of the brain with leaky vasculature. This suggestion is supported by the finding that vessels of the subventricular zone (SVZ) are leaky, allowing direct communication between resident cells of this niche and humoral components (Tavazoie et al., 2008). However, effects of CCL11 were only characterized in a distinct neurogenic niche, the subgranular zone (SGZ) of the hippocampus (Villeda et al., 2011). Differences in vascular architecture and function are observed in the SGZ versus SVZ (Goldberg and Hirschi, 2009), raising the question of whether these regions are equally permeable to factors in the circulation.

An unexplored possibility is that CCL11 crosses the blood-brain barrier (BBB). One of the many functions of the BBB is to serve as a transport interface for molecules in and out of the brain. Cytokines are one category of molecules transported across the BBB (Banks, 2005), and this process is important for aspects of the neuroinflammatory response, such as memory impairments that are a feature of sickness behavior

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ABBREVIATIONS: BBB, blood-brain barrier; CCL11, C-C motif ligand 11; CCR3, C-C motif ligand 3; CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; Expt, exposure time; LR, lactated Ringer's solution; MBEC, mouse brain capillary endothelial cell; MS, multiple sclerosis; PBS, phosphate-buffered saline; SB328437, *N*-(1-naphthalenylcarbonyl)-4-nitro-L-phenylalanine methyl ester; SGZ, subgranular zone; SVZ, subventricular zone; TCA, trichloroacetic acid; TEER, transendothelial electrical resistance.

(Banks et al., 2002–2003). In this study, we characterized transport of CCL11 across the BBB. Our major findings were that CCL11 crosses the BBB, transport occurs throughout the brain, transport rates vary among brain regions, and transport is affected by interactions with the cellular components of blood. These findings suggest that CCL11 in the blood is a source of CCL11 in the brain, and at physiologic levels may have important functions in the CNS.

Materials and Methods

Animal Subjects. Male CD-1 mice were purchased from Charles River Laboratories (Seattle, WA), housed on a 12/12-hour light/dark cycle, and provided food and water ad libitum. Experiments were conducted when the mice were between 7 and 8 weeks of age. All studies were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and approved by the animal committee of the Veterans Administration Medical Center.

Iodination of CCL11 and Albumin. Murine CCL11 was purchased from R&D Systems (Minneapolis, MN) and bovine serum albumin (albumin) from Sigma-Aldrich (St. Louis, MO). CCL11 was radioactively labeled with ^{125}I by the chloramine-T method. In brief, 5 μg of CCL11 was incubated with 0.5 mCi of ^{125}I (carrier-free) and 10 μg of chloramine-T for 1 minute in 0.25 M phosphate buffer, pH 7.5. The reaction was terminated by adding 100 μg of sodium metabisulfite, followed by purification on a G-10 Sephadex column. Specific activity of iodinated CCL11 was approximately 8.25 Ci/g. Albumin was labeled with 2 mCi of ^{131}I by the same method. Protein labeling by iodine isotopes was characterized by trichloroacetic acid (TCA) precipitation. Greater than 90% radioactivity in the precipitated fraction was consistently observed for CCL11 and albumin.

Measurement of Iodinated CCL11 Uptake by Mouse Brain. To determine the kinetics of brain uptake, mice were anesthetized with intraperitoneal urethane and given an intravenous bolus injection of 300,000 cpm (approximately 20 ng) ^{125}I -CCL11 into the left jugular vein. In some experiments, 150,000 cpm of ^{131}I -albumin were coinjected to quantify vascular space. After a circulation time ranging from 1 to 30 minutes, serum and whole brains or brain regions were collected and counted in a Wizard2 automatic gamma counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Multiple time regression analysis (Blasberg et al., 1983; Kastin et al., 2001) was used to calculate the blood-to-brain unidirectional influx rate (K_i). The brain/serum ratios were plotted against their respective exposure times (Expt). Expt was calculated from the formula:

$$\text{Expt} = \left(\int_0^t C_p(t) dt \right) / C_{pt}$$

where C_p is the level of radioactivity in serum and C_{pt} is the level of radioactivity in serum at time t . Expt corrects for the clearance of peptide from the blood. K_i , with its error term, is measured as the slope for the linear portion of the relation between the brain/serum ratios and Expt, and the y -intercept of the linearity measures the distribution volume (V_d) in brain at $t = 0$. Because two linear phases with different slopes were observed for CCL11 uptake, K_i was calculated for two clock-time intervals: 1–10 minutes and 10–30 minutes. Saturability of ^{125}I -CCL11 uptake was determined by coinjecting 1 μg of unlabeled CCL11. Effects of CCL11 on its own transport were also tested by injecting 20 ng of unlabeled CCL11 10 minutes prior to the injection of ^{125}I -CCL11 and by injection of a very low dose (one-tenth the dose used in the standard experiment) of ^{125}I -CCL11 and of ^{131}I -albumin (30,000 and 15,000 cpm, respectively).

The percentage of the intravenously injected dose taken up into each gram of brain tissue (%inj/g) was calculated by multiplying the parenchymal values (in microliters per gram) by the percentage of the injected dose found in a microliter of blood. Activity in vascular space

was excluded by subtracting the brain/serum ratios of ^{131}I -albumin from the brain/serum ratios of ^{125}I -CCL11.

Characterization of Radioactivity Extracted from Brain and Blood in Mice. Radioactivity recovered from brain tissue and serum was characterized by TCA precipitation. Whole brains and serum from mice that had received ^{125}I -CCL11 were harvested on ice at 5, 15, or 30 minutes postinjection. Processing controls were generated by spiking radioactivity into collection tubes and harvesting organs from mice not receiving a radioactive injection. Brains were homogenized in 3 ml of ice-cold lactated Ringer's solution (LR) containing 1% albumin using a glass tissue grinder and seven vertical strokes of the pestle. The homogenate was centrifuged at 10,000g for 20 minutes, and the supernatant was collected. For acid precipitation of brain tissue, equal volumes (1.5 ml) of supernatant from the homogenate and 30% TCA were mixed. For acid precipitation of serum, 50 μl of serum was mixed with 250 μl of 1% albumin/LR, and proteins were precipitated by adding 300 μl of 30% TCA. Precipitates were centrifuged at 5400g for 10 minutes, and the pellets and supernatants were counted in a gamma counter. The percentage of radioactivity in the pellet reflects intact protein.

Cellular Partitioning of CCL11. Partitioning of CCL11 into plasma/cellular fractions was determined ex vivo using an adapted method from Hinderling (1997). Whole blood was collected from the carotid arteries of male CD-1 mice into tubes containing EDTA or citrate, and 50- μl aliquots were prepared. All tubes were centrifuged at 1000g, and the meniscus of the plasma layer was marked on each tube. For some aliquots, plasma was removed and the cellular fraction was washed three times in phosphate-buffered saline (PBS). The plasma volume was then replaced with an equivalent volume of PBS with or without anticoagulant, and 1000/500 cpm of ^{125}I -CCL11/ ^{131}I -albumin were mixed with each aliquot and incubated for 5 minutes at 37°C. Samples were then placed on ice immediately for 1 minute and centrifuged for 5 minutes at 1000g and 4°C. PBS/plasma fractions were separated from cellular fractions, and both were counted on a gamma counter.

In Situ Brain Perfusion. In situ brain perfusions were performed as described previously (Banks et al., 2000). Mice were anesthetized with intraperitoneal urethane, the thorax was opened, and the heart was exposed. Both jugular veins were severed, and the descending thoracic aorta was clamped. A 26-gauge butterfly needle was inserted into the left ventricle of the heart, and Zlokovic's buffer (7.19 g/l NaCl, 0.3 g/l KCl, 0.28 g/l CaCl_2 , 2.1 g/l NaHCO_3 , 0.16 g/l KH_2PO_4 , 0.17 g/l anhydrous MgCl_2 , and 0.99 g/l D -glucose, with 10 g/l bovine serum albumin added on the day of perfusion) containing 60,000/30,000 cpm/ml ^{125}I -CCL11/ ^{131}I -albumin was infused at a rate of 2 ml/min for 1–25 minutes. After perfusion, the needle was removed and the mouse was decapitated. Brain/perfusion ratios were calculated by dividing the counts per minute by brain weight in grams and by the counts per minute in 1 μl of perfusion fluid to yield units of microliters per gram. In some experiments, various concentrations of freshly prepared CCR3 inhibitor *N*-(1-naphthalenylcarbonyl)-4-nitro-L-phenylalanine methyl ester (SB328437) (Tocris Bioscience, Bristol, UK) or vehicle (dimethylsulfoxide) were included. Perfusions in these experiments were for 15 minutes. The perfused brain remains viable for approximately 7 hours (Kriegelstein et al., 1972), and the BBB remains intact until approximately 12 hours after death (Broman et al., 1950; Grontoft, 1954).

Capillary Depletion in Mice. To determine whether CCL11 completely crossed the BBB, we performed capillary depletion as adapted to mice (Triguero et al., 1990; Gutierrez et al., 1993). Mice anesthetized with intraperitoneal urethane received an intravenous injection of 300,000 cpm of ^{125}I -CCL11. Either 10 or 30 minutes after intravenous injection, blood from the abdominal aorta was collected. The thorax was then opened, the descending thoracic aorta clamped, the jugular veins severed, and the vascular space of the brain washed free of blood by perfusing 20 ml of LR through the left ventricle of the heart. The brain was removed, weighed, and homogenized with seven strokes of a glass homogenizer in 0.8 ml of physiologic buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl_2 , 1 mM MgSO_4 ,

1 mM NaH_2PO_4 , and 10 mM D-glucose adjusted to pH 7.4). An equal volume of 40% dextran solution was added to the homogenate, which was thoroughly mixed by inverting 10 times. Homogenates were centrifuged at 3500g for 20 minutes at 4°C in a Beckman Allegra 21R centrifuge with a swinging bucket rotor (Beckman Coulter, Inc., Fullerton, CA). The pellet containing the brain vasculature and the supernatant containing the brain parenchyma were carefully separated, and the level of ^{125}I was determined in a gamma counter. The fractions were expressed as percentages of total activity in the brain.

Measurement of Brain-to-Blood Efflux. A method previously described that accurately quantifies efflux rates was used (Banks and Kastin, 1989). Mice were anesthetized with intraperitoneal urethane, the scalp was removed, and a hole was made through the cranium 1.0 mm lateral and 1.0 mm posterior to the bregma with a 26-gauge needle. Tubing covered all but the terminal 2.5–3.0 mm of the needle so that the tip of the needle penetrated the brain tissue forming the roof of the lateral ventricle but did not penetrate its floor. One microliter of LR-albumin containing 20,000/10,000 cpm of ^{125}I -CCL11/ ^{131}I -albumin was injected into the lateral ventricle with a 1- μl Hamilton syringe. To determine the rate of efflux, three mice were decapitated at each time of 2, 5, 10, 20, and 30 minutes, and the residual radioactivity in the brain was determined. The amount of radioactivity in the brain at $t = 0$ was estimated as previously described in mice that had been overdosed with urethane. The log of the percentage of injected counts remaining in the whole brain was plotted against time, and the slope was used to calculate the half-time disappearance rate. To determine whether there was a saturable component to the retention of CCL11 by the brain after intracerebroventricular injection, other mice received an intracerebroventricular injection containing 20,000 cpm of ^{125}I -CCL11 and 1 μg of unlabeled CCL11. The mice were decapitated 10 minutes after injection, and the residual radioactivity was expressed as the percentage of the injection remaining in the brain.

CCL11 Enzyme-Linked Immunosorbent Assay in Brain and Blood. Mice received an intravenous bolus of 1 μg of CCL11 in 100 μl of LR + 1% albumin or vehicle, and brains and serum were collected 10 and 30 minutes after injection as described under “Capillary Depletion in Mice.” Immediately after collection, hemibrains and serum were snap-frozen in liquid nitrogen. Frozen hemibrains were homogenized in 1 ml of ice-cold PBS plus 1mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich). Triton X-100 was added to a final concentration of 0.1% after homogenization, and samples were vortexed briefly and centrifuged at 20,000g for 10 minutes at 4°C. Supernatants were collected and stored at -80°C until assay. CCL11 levels were quantified in brain tissue and serum using an ELISA kit for murine CCL11 from R&D Systems (Minneapolis, MN). Samples were diluted in calibrator diluent provided in the kit, and assayed according to kit instructions. CCL11 concentrations in brain extracts were normalized to total protein, which was determined using a BCA assay kit (Thermo Fisher Scientific, Waltham, MA). For estimation of tissue/serum ratios, CCL11 measurements in brain tissue (in units of pictograms per milliliter) were first multiplied by 2 to estimate the amount of CCL11 extracted from a whole brain. This value was then divided by 0.475 g, which approximates the average brain weight of mice used in this study. This value was divided by measurements of CCL11 in serum (in units of picograms per microliter) to give final units of microliters per gram.

In Vitro Blood-Brain Barrier Model. Primary cultures of mouse brain capillary endothelial cells (MBECs) were isolated from 8-week-old male CD1 mice according to published protocols (Coisne et al., 2005) with modifications. MBECs were seeded on dishes (flasks, plates) coated with collagen type IV and fibronectin (both 0.1 mg/ml). MBEC cultures were maintained in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% plasma-derived serum (PDS, Animal Technologies, Inc., Tyler, TX), 1% GlutaMAX supplement (Gibco, St. Louis, MO), basic fibroblast growth factor (1 ng/ml; Roche Applied Sciences, Indianapolis, IN), heparin (100 $\mu\text{g}/\text{ml}$), insulin (5 $\mu\text{g}/\text{ml}$), transferrin (5 $\mu\text{g}/\text{ml}$), sodium selenite (5 ng/ml) (insulin-transferrin-sodium-selenite

media supplement), and gentamicin (50 $\mu\text{g}/\text{ml}$) at 37°C with a humidified atmosphere of 5% $\text{CO}_2/95\%$ air; pericytes were eliminated from the culture by including puromycin (4 $\mu\text{g}/\text{ml}$) (Perriere et al., 2005) in this medium (MBEC medium 1). Red blood cells, cell debris, and nonadherent cells were removed 24 hours after plating by washing with medium. On the third day, the cells received a new medium that contained all components of MBEC medium 1, with the exception of puromycin (MBEC medium 2). When the cultures reached 80% confluence (fifth day in vitro), the purified endothelial cells were passaged by brief treatment with 0.25% trypsin-EDTA (Gibco) solution

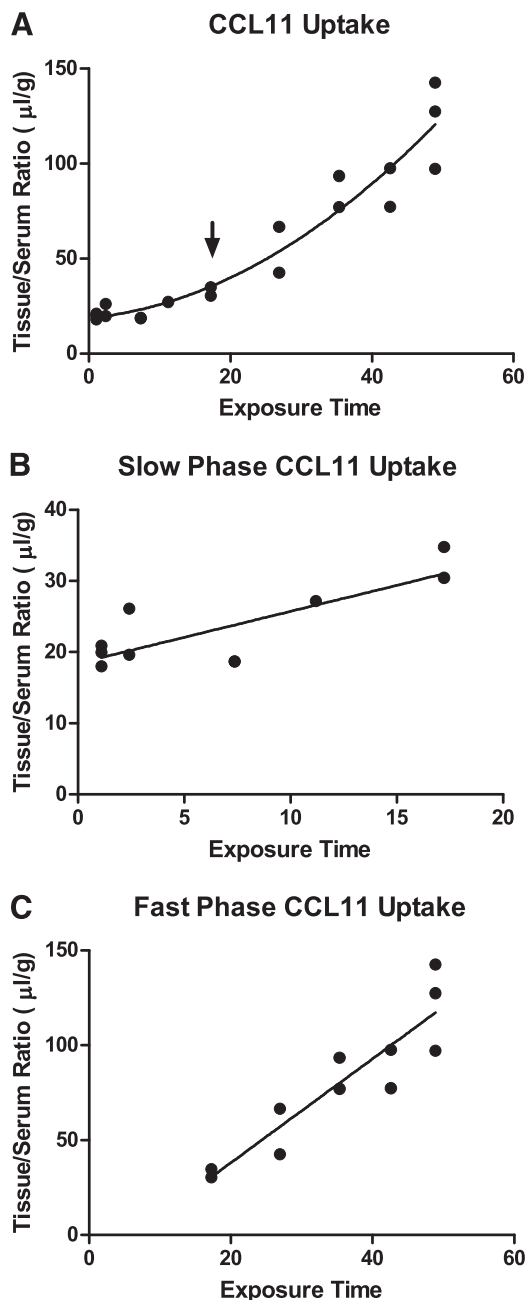


Fig. 1. CCL11 influx into the brain. Slow and fast phases of uptake are shown in the full time curve (A). The arrow (10 minutes clock time) marks the inflection point of the curve. The slow (B) and fast (C) phases are plotted between 1–10 minutes clock time and 10–30 minutes clock time. Unidirectional influx constants for the slow and fast phases are $0.7296 \pm 0.1894 \mu\text{l}/\text{g}$ per min ($r^2 = 0.6498$, $df = 8$) and $2.735 \pm 0.4068 \mu\text{l}/\text{g}$ per min ($r^2 = 0.8340$, $df = 9$), respectively. Both slopes are significantly non-zero.

and seeded on 24-well plate-formatted Transwell inserts (Corning Inc., Corning, NY) at a density of 40,000 cells per insert. To determine CCL11 effects on transendothelial electrical resistance (TEER), 0–100 ng/ml CCL11 was applied to the luminal chamber for 4 hours. After treatment, TEER was measured using an EVOM voltohmmeter equipped with an STX-2 electrode (World Precision Instruments, Sarasota, FL). Readings were calculated as Ωcm^2 , and resistance of a Transwell insert without cells was subtracted from all monolayer readings.

Statistics. Means are reported with their standard errors and were compared by analysis of variance followed by Newman-Keuls or Dunnett's multiple comparisons test for one-way models, and Bonferroni post-tests for two-way models. Regression lines were computed by the least-squares method and compared statistically with the Prism 5.0 program (GraphPad Software, Inc., San Diego, CA).

Results

Characterization of BBB Influx of CCL11. Following multiple-time regression analysis, CCL11 was found to have a two-phased curve of uptake into brain tissue. The slow phase occurred up to 10 minutes postinjection (Fig. 1, A and B; $K_i = 0.7296 \pm 0.1894 \mu\text{l/g}$ per min, $r^2 = 0.6498$, $df = 8$) followed by a rapid rate of uptake (Fig. 1, A and C; $K_i = 2.735 \pm 0.4068 \mu\text{l/g}$ per min, $r^2 = 0.8340$, $df = 9$). Both slopes were significantly non-zero. Coinjection of $1 \mu\text{g}$ of unlabeled CCL11 had no significant effects on the K_i of ^{125}I -CCL11 or ^{131}I -albumin (Fig. 2; Table 1), which demonstrates that the transport system for CCL11 is either nonsaturable or high capacity and that even high doses of CCL11 do not disrupt the BBB within the time period studied. The absence of effects of CCL11 on BBB disruption is further supported by *in vitro* findings. No change in TEER was observed after CCL11 addition to the luminal chamber at concentrations ranging approximately from 2 to 200 times higher than those occurring in serum (Fig. 3). We found that neither 10-minute preinjection of a comparable amount (20 ng) of unlabeled CCL11 nor injection of one-tenth of the cpm of ^{125}I -CCL11 significantly affected the K_i of the slow or fast phases of transport (Fig. 4; Table 2). This demonstrates that CCL11 does not activate its own transport in a time-dependent manner, nor does it inhibit its own transport in a concentration-dependent manner. To confirm that activity measured in brain tissue and serum represents intact ^{125}I -CCL11 and not free iodine, serum and brain homogenates were acid precipitated at different time points following intravenous injection of ^{125}I -CCL11. Although significant levels of free ^{125}I were measured in serum beginning at 15 minutes postinjection (Fig. 5A), the proportion of labeled peptide remained greater than 60% for the duration of the uptake study. Furthermore, the unidirectional influx constants did not significantly differ when free ^{125}I was excluded from

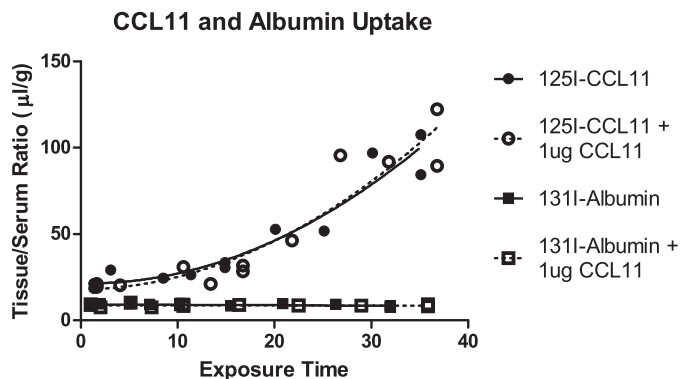


Fig. 2. Saturability of CCL11 influx after intravenous injection. Curves show CCL11 (circles) and albumin (squares) in the presence (unfilled, dotted line) or absence (filled, continuous line) of $1 \mu\text{g}$ of CCL11.

serum calculations (data not shown). The half-life of intact ^{125}I -CCL11 in serum was found to be approximately 4 minutes. Activity in brain tissue was predominantly intact ^{125}I -CCL11, and no time-dependent increases in free iodine occurred (Fig. 5B). Brain perfusion of CCL11, which excludes influence of blood factors on brain uptake, showed no slow phase of uptake, and brain uptake of CCL11 became non-linear after 15 minutes of perfusion (Fig. 6). Notably, the albumin uptake curve remained flat for the duration of perfusion, indicating an intact BBB. This is consistent with a prior report of preserved BBB integrity for up to 30 minutes of *in situ* perfusion (Greenwood et al., 1985). The K_i for the linear portion of the curve ($3.797 \pm 0.5567 \mu\text{l/g}$ per min, $r^2 = 0.8231$, $df = 10$) was comparable to those calculated for the fast portion of uptake curves following intravenous injection. This suggests that blood factors contribute to the slow phase of CCL11 transport.

Ex vivo studies revealed that CCL11 significantly partitions into the cellular fraction of citrated blood compared with albumin (Fig. 7). Plasma proteins did not affect the cellular partitioning of CCL11 in citrated blood, as proven by the absence of significant difference in partitioning when citrated plasma was replaced with PBS with or without citrate (Fig. 7A). When this study was repeated with EDTA replacing citrate as the anticoagulant, cellular partitioning was also significantly elevated compared with albumin (Fig. 7B), but EDTA had a suppressive effect on cellular partitioning compared with citrate (Fig. 7, B and C). These findings suggest that cellular components of blood may be reservoirs for circulating CCL11, and that anticoagulants may influence results of CCL11 measurement in plasma.

To validate the findings that used iodinated CCL11, we measured brain and serum levels of CCL11 by enzyme-linked immunosorbent assay (ELISA) 10 and 30 minutes after an intravenous bolus of $1 \mu\text{g}$ of unlabeled CCL11. At 10 minutes

TABLE 1

Determination of CCL11 transport saturability

Unlabeled CCL11 had no statistically significant effect on ^{125}I -CCL11 transport across the BBB.

	Slow Phase			Fast Phase		
	K_i	r^2	df	K_i	r^2	df
^{125}I -CCL11 only	0.6762 ± 0.2468	0.6002	5	3.301 ± 0.5585	0.8748	5
^{125}I -CCL11 + $1 \mu\text{g}$ of CCL11	0.5992 ± 0.2372	0.5608	5	3.864 ± 0.7296	0.8487	5

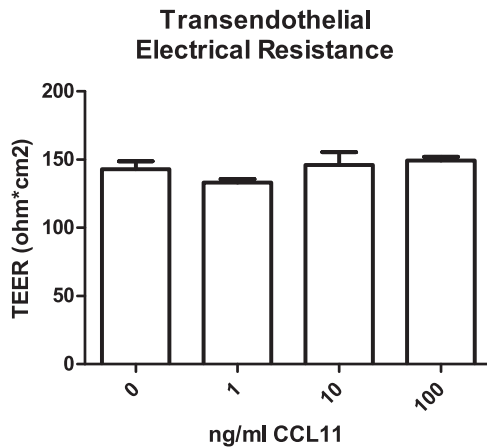


Fig. 3. CCL11 effects on BBB disruption in vitro. Monolayers of mouse brain endothelial cells were treated with varying doses of CCL11 for 4 hours. TEER values post-treatment are shown. Data were analyzed by one-way analysis of variance with Newman-Keuls multiple comparison test; $n = 5$ per group.

postinjection, the concentration of CCL11 in the brain was more than twice that of endogenous levels (Fig. 8A), and this concentration remained elevated in brain tissue at 30 minutes despite substantial serum clearance by this time (Fig. 8, A and B). To compare tissue/serum ratios at steady state, we estimated the picograms of CCL11 per gram of brain tissue as described under *Materials and Methods* for the control group. The mean ratio was $85.30 \pm 10.68 \mu\text{l/g}$, which is comparable to the plateau of the perfusion curve shown in Fig. 6 ($89.86 \pm 13.25 \mu\text{l/g}$). Therefore, brain uptake of CCL11 occurs as assessed by both radioactive and immunoactive methods.

To confirm that CCL11 completely traverses the BBB into the brain parenchyma, capillary depletion studies were performed at 10 and 30 minutes after intravenous injection of ^{125}I -CCL11. At 10 minutes postinjection, 60% of the ^{125}I -CCL11 was present in the capillary-depleted parenchymal fraction (Fig. 9). By 30 minutes postinjection, this proportion increased to 85% (Fig. 9). This demonstrates that CCL11 completely traverses the brain vasculature and enters the brain parenchyma in a time-dependent manner.

Characterization of BBB Efflux of CCL11 from Brain.

Efflux of ^{125}I -CCL11 was determined by measuring its disappearance from brain tissue at multiple time points following

injection into the lateral ventricle of the brain. ^{131}I -Albumin was coinjected as a marker for cerebrospinal fluid reabsorption. The relation between the log %inj/brain versus time for CCL11 (Fig. 10A, $m = -0.002811 \pm 0.001323$, $r^2 = 0.1388$, $df = 28$) was more than seven times slower than that of albumin (Fig. 10B, $m = -0.02002 \pm 0.002256$, $r^2 = 0.773$, $df = 28$), indicating that sequestration of CCL11 in the brain precludes its efflux. However, the slopes of both relations were significantly non-zero, supporting that an efflux system for CCL11 is present at the BBB. Furthermore, coinjection of $1 \mu\text{g}$ of unlabeled CCL11 significantly inhibited efflux of CCL11 (Fig. 10B), demonstrating that CCL11 efflux is saturable.

Regional BBB Influx of CCL11. To determine whether BBB transport of CCL11 varies by brain region, we measured ^{125}I -CCL11 uptake in 11 brain regions: olfactory bulb, striatum, frontal cortex, hypothalamus, hippocampus, thalamus, parietal cortex, occipital cortex, cerebellum, midbrain, and pons-medulla. ^{131}I -Albumin was coinjected for vascular space correction. Unidirectional influx constants were calculated between 1–10 minutes and 10–30 minutes to determine whether slow and fast phases were detectable and/or variable between brain regions; results are shown in Table 3. Slopes calculated up to 10 minutes were significantly positive for striatum, parietal cortex, occipital cortex, and cerebellum. Slopes calculated between 10 and 30 minutes were significantly positive for all brain regions except for striatum. Regional uptake was also calculated as a percentage of injected activity per gram of tissue, and the mean uptake at 10 and 30 minutes for each region is shown in Fig. 11. Consistent with uptake kinetics, striatum had the highest amount of CCL11 entering the brain by 10 minutes, and this intake was significantly higher than all other brain regions (Fig. 11A). The region showing the next highest level of CCL11 uptake at 10 minutes was the hypothalamus, which was significantly higher than frontal, parietal, and occipital cortices; thalamus; midbrain; and pons-medulla (Fig. 11A). No other significant differences in uptake were observed among brain regions by 10 minutes. By 30 minutes, CCL11 uptake was significantly increased in all groups except striatum and parietal cortex, which showed a decrease and no change, respectively, when compared with uptake at 10 minutes. No significant differences in uptake were evident among regions in the 30-minute group (Fig. 11B). Unidirectional influx constants for ^{131}I -albumin showed no significant uptake for any brain region, nor did %inj/g significantly differ

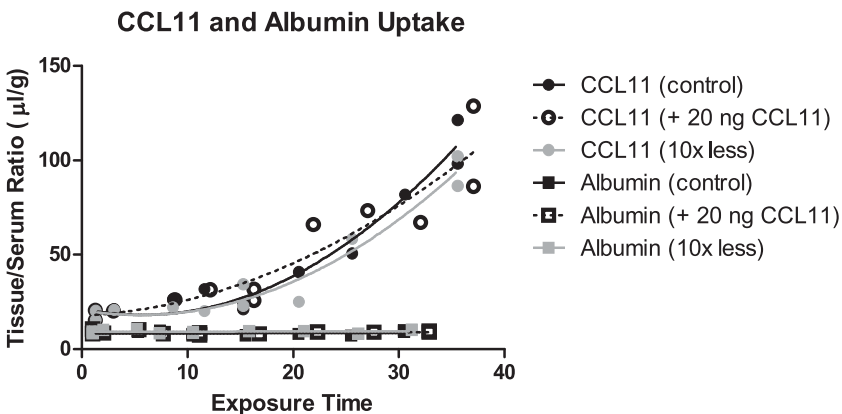


Fig. 4. Short-term effects of CCL11 on self-transport. Effects of a 10-minute preinjection of uniodinated CCL11 (unfilled circles/squares and dotted lines) or injection of 10-fold less ^{125}I -CCL11 (gray-filled circles/squares and gray continuous lines) on ^{125}I -CCL11 (circles)/ ^{131}I -albumin (squares) uptake into the CNS. Controls are black-filled circles/squares and black continuous lines.

TABLE 2
Short-term effects of CCL11 on its own transport

	Slow Phase			Fast Phase		
	K_i	r^2	df	K_i	r^2	df
Control	0.5662 ± 0.3148	0.3928	5	4.249 ± 0.4614	0.9443	5
20 ng of Preinjection	0.7822 ± 0.2125	0.7304	5	3.399 ± 0.8064	0.7804	5
10× Fewer cpm	0.6478 ± 0.2987	0.4848	5	3.416 ± 0.5430	0.9082	4

among brain regions at 10 or 30 minutes (data not shown). The distribution volumes of ^{131}I -albumin at $t = 0$ are shown in Fig. 11C. Olfactory bulb, pons-medulla, and cerebellum showed the highest distribution volumes of albumin. Significant differences were observed between olfactory bulb and all other brain regions; between pons-medulla and hypothalamus, parietal cortex, striatum, hippocampus, thalamus, occipital cortex, frontal cortex, and midbrain; and between cerebellum and hypothalamus.

Effects of CCR3 Inhibition on CCL11 Uptake. The predominant receptor for CCL11 is CCR3, whose expression has been detected on brain endothelial cells (Mukhtar et al., 2002; Khan et al., 2007). To determine whether CCR3 participates in CCL11 uptake, we coperfused ^{125}I -CCL11 with

different concentrations of the CCR3 inhibitor SB328437. Although a low concentration of inhibitor caused a nonsignificant decrease in the mean tissue/perfusate ratio, the highest concentration caused a significant increase in ^{125}I -CCL11 uptake (Fig. 12A). ^{131}I -Albumin was coperfused in all groups and showed no significant changes in tissue/perfusate ratio compared with control at any concentration of inhibitor (Fig. 12B). These findings indicate that CCR3 is not a predominant transporter of CCL11 at the BBB, although it may compete with the CCL11 transport system for ligand binding or participate in brain-to-blood efflux.

Discussion

The findings of Villeda et al. (2011) were the first to clearly demonstrate a function of CCL11 in the CNS. This study also showed that antineurogenic effects of CCL11 occur as a result of age-related increases in blood levels of CCL11. Adult neurogenesis is thought to be important in the preservation of cognitive function (Kempermann et al., 2004). Of note, other conditions associated with cognitive decline, such as obesity (Elias et al., 2003) and depression (Jorm, 2000), have also been associated with increased levels of CCL11 in the circulation (Vasudevan et al., 2006; Grassi-Oliveira et al., 2012). Although previous findings suggest that CCL11 from the circulation has direct effects on the neurogenic niche (Villeda et al., 2011), the mechanism by which CCL11 affects the brain has not been determined. Here, we have characterized CCL11 in the context of its ability to cross the BBB. Our main findings were that 1) CCL11 influx supports its accumulation in the CNS, 2) CCL11 efflux is slow but saturable, and 3) fast rates of CCL11 uptake

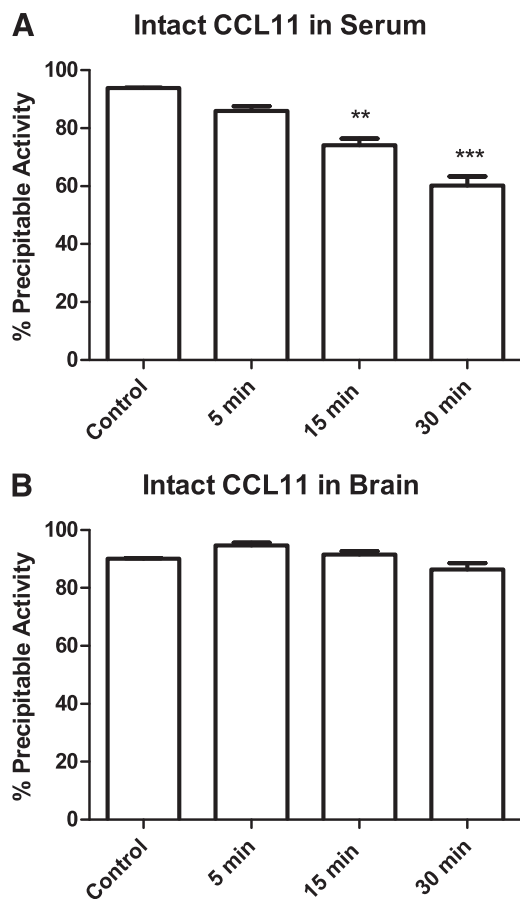


Fig. 5. Acid precipitation of ^{125}I -CCL11 from brain and serum. Intact ^{125}I -CCL11 was determined in serum (A) and brain (B) at 5, 15, and 30 minutes after intravenous injection. Controls reflect loss of label due to processing. Data were analyzed by one-way analysis of variance with Dunnett's multiple comparisons test, $n = 2$ or 3 per group. ** $P < 0.01$; *** $P < 0.001$ versus control.

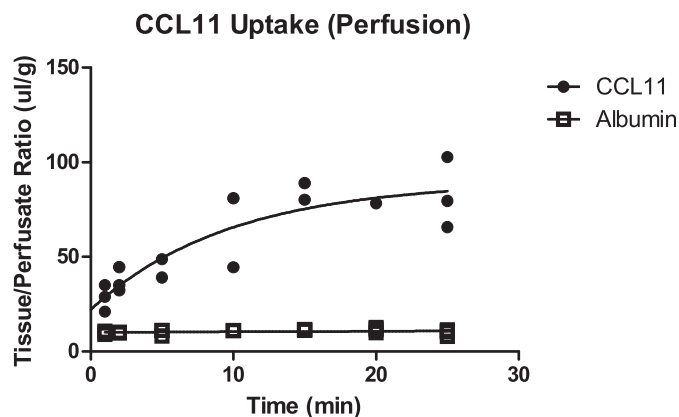


Fig. 6. In situ brain perfusion of ^{125}I -CCL11 (circles) and ^{131}I -albumin (squares). Linear uptake was observed up to 15 minutes. For CCL11, the K_i for the linear portion of the curve was $3.797 \pm 0.5567 \mu\text{l/g per min}$ ($r^2 = 0.8231$, $df = 10$). The slope for CCL11 but not for albumin was significantly non-zero, formally demonstrating BBB transport.

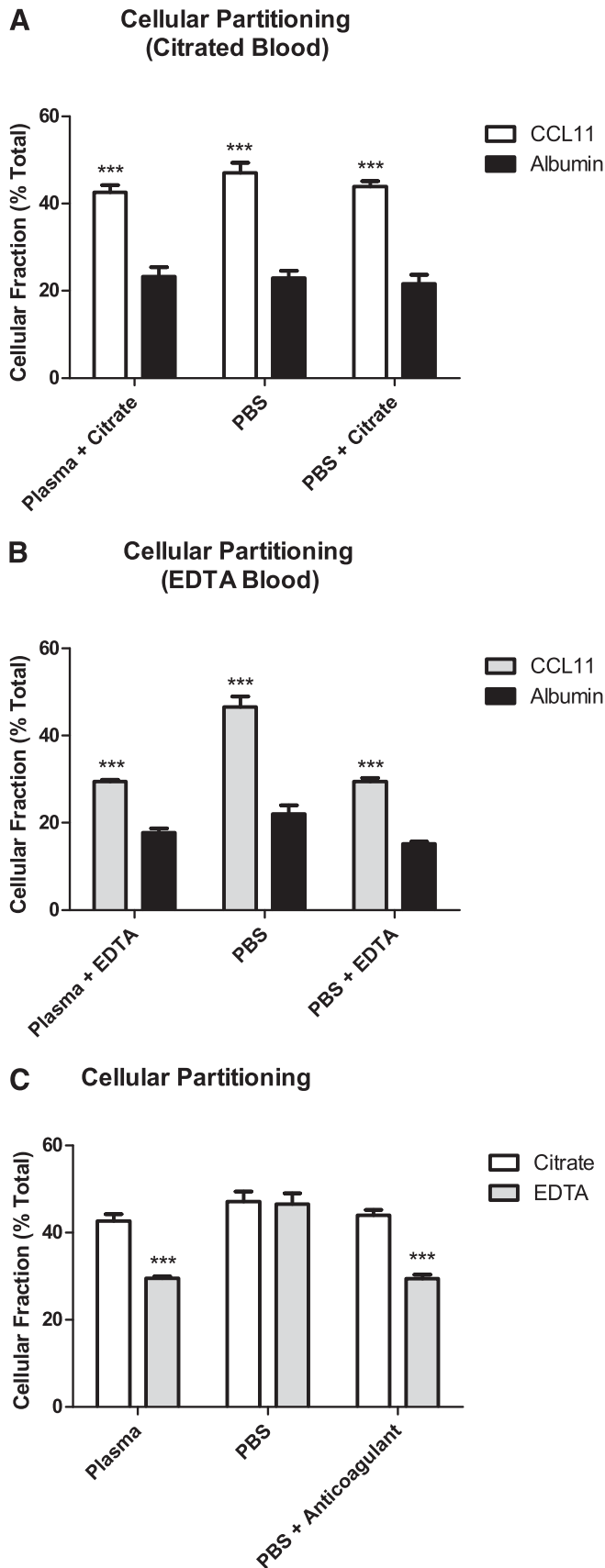


Fig. 7. Cellular partitioning of ^{125}I -CCL11 and ^{131}I -albumin. Partitioning of CCL11 into the plasma and cellular fractions of blood ex vivo using citrate (A and C) or EDTA (B and C) as anticoagulants. Effects of EDTA versus citrate are compared in (C). Data were analyzed by two-way

analysis of variance with Bonferroni post-test, $n = 4$ per group. *** $P < 0.001$ versus albumin (A and B) or versus citrate (C); ### $P < 0.001$ versus CCL11/PBS (B); @@ $P < 0.01$ versus albumin/PBS (B).

are observed in most brain regions tested. These three findings support the suggestion that the BBB contributes to processes governing CCL11 entry into the CNS and its subsequent inhibitory effects on neurogenesis and cognitive function.

Influx of CCL11 into the brain revealed a slow phase of influx up to 10 minutes, followed by a rapid phase of influx between 10 and 30 minutes. Uptake kinetics were not explained by the ^{125}I -CCL11 bolus either activating or inhibiting its own transport. They were also not explained by disruption of the BBB, as neither coinjection in vivo with a high dose of CCL11 nor CCL11 treatment of an in vitro brain endothelial monolayer model of the BBB caused appreciable changes in albumin influx or TEER, respectively.

Blood cell partitioning can have important effects on the pharmacokinetics and dynamics of circulating molecules (Hinderling, 1997). For example, partitioning of circulating amino acids and glucose into red blood cells influences their uptake by the brain (Drewes et al., 1977; Jacquez, 1984). We found that CCL11 partitioned into the cellular fraction of blood ex vivo. Furthermore, exclusion of blood components by in situ brain perfusion resulted in uptake kinetics similar to the fast rate of transport for the linear phase. These ex vivo and in situ findings suggest that binding of CCL11 to cellular components in blood could mediate the slow phase of transport into the CNS. The fast phase could reflect a tradeoff of CCL11 from binding sites in blood to binding sites on brain endothelial cells. We also found that the degree of cellular partitioning in blood was affected by the type of anticoagulant used. Heparin was not tested because of previous findings that CCL11 binds to heparin (Ellyard et al., 2007). EDTA was found to cause reduced partitioning of CCL11 into the cellular fraction compared with PBS without anticoagulant or with citrate, suggesting that divalent metals facilitate binding of CCL11 to blood cells. CCL11 partitioning in citrated plasma or PBS was similar to partitioning observed in citrate-free PBS, indicating that citrate did not influence cellular partitioning. Therefore, CCL11 measurements in plasma could vary based on use of an anticoagulant. Because of growing interest in CCL11 as a biomarker, this finding represents an important methodological consideration for subsequent studies. Together, these results suggest that cellular reservoirs of CCL11 in blood play a regulatory role in the brain's uptake of CCL11.

Neither the slow or fast phases of CCL11 uptake were saturated when a 50-fold excess of CCL11 was coinjected. We found that this dose of CCL11 is sufficient to increase serum levels by greater than 15-fold and 6-fold physiologic levels at 10 and 30 minutes postinjection, respectively. It is noteworthy that these increases exceed the 300% increase in CCL11 reported in aged mice (Villeda et al., 2011). This indicates that the CCL11 transport system would not be saturated at physiologic ranges in aging. We also found that ^{125}I -CCL11 completely traverses the BBB into brain parenchyma and remains intact once in the brain. These results are not likely to be caused by labeling artifacts, as unlabeled CCL11 in the circulation also enters the brain at a rapid rate. Increases of

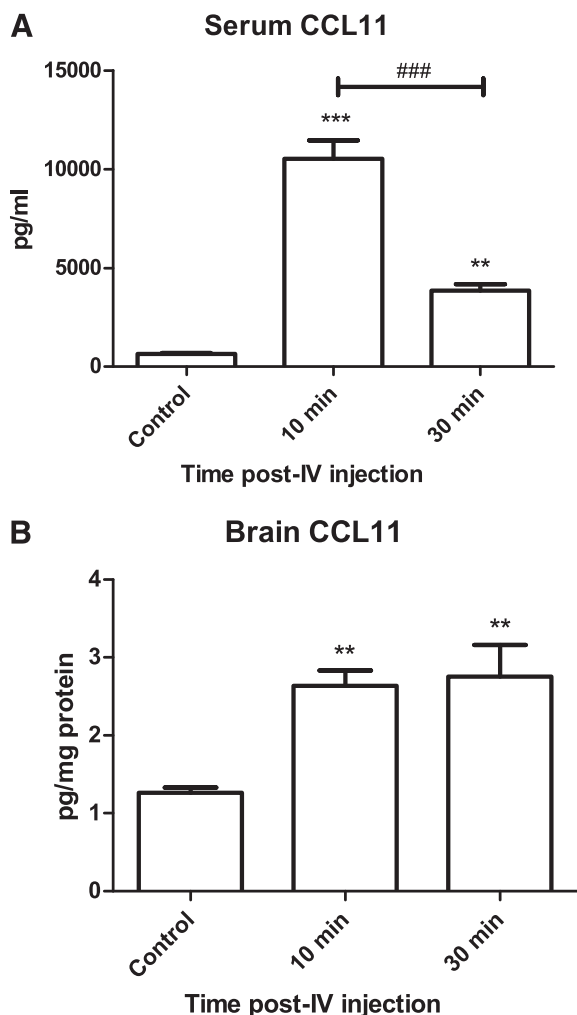


Fig. 8. ELISA measurement of unlabeled CCL11 transport. 1- μ g Unlabeled CCL11 was injected intravenous, and brain and serum were harvested 10 or 30 minutes postinjection. CCL11 levels in serum (A) and brain (B) were measured by ELISA at 10 and 30 minutes postinjection. Data were analyzed by one-way analysis of variance with Newman-Keuls multiple comparisons test, $n = 6$ per group. $**P < 0.01$; $***P < 0.001$ versus control; $###P < 0.001$ versus group indicated.

unlabeled CCL11 in brain tissue are observed by 10 minutes, which is unlikely to be explained by de novo synthesis of CCL11 in the brain and is therefore reflective of entry. These data strongly suggest that CCL11 crosses the BBB at levels sufficient to promote accumulation in the CNS.

Efflux systems at the BBB have been characterized for compounds whose accumulation in the CNS would lead to neuronal damage such as amyloid β (Shibata et al., 2000), prostaglandin E_2 (Akanuma et al., 2010), and interleukin-2 (Banks et al., 2004). We observed that an efflux system also exists for CCL11. Efflux of CCL11 from the brain was slower than efflux of albumin, which is a marker of clearance by cerebrospinal fluid. This suggests that binding sites for CCL11 in the CNS compete with CCL11 efflux. Despite this, we observed that efflux of CCL11 from the brain was saturable. Therefore, the efflux system for CCL11 may reflect a mechanism by which the CNS buffers against unbound CCL11 that could otherwise be harmful in regions such as the neurogenic niche.

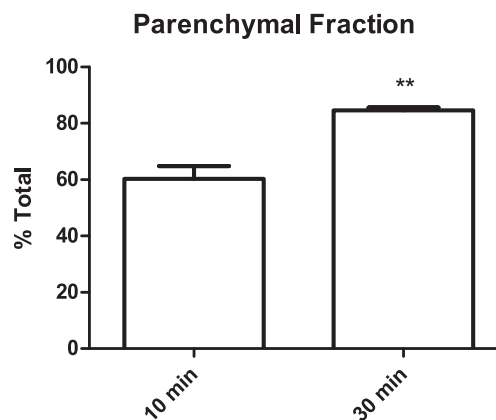


Fig. 9. Capillary depletion. Uptake of CCL11 into the vascular and parenchymal fractions of the brain were determined at 10 and 30 minutes after intravenous injection of 125 I-CCL11. Data are expressed as the percentage of total radioactivity (vascular + parenchymal) in the parenchymal fraction. Data were analyzed by two-tailed unpaired t test, $n = 3$ per group. $**P < 0.01$.

To determine whether CCL11 uptake was specific for certain brain regions, we measured uptake of CCL11 in 11 regions of the brain. Surprisingly, we found that most brain regions had slow and fast phases of uptake with the exception of striatum. The striatum showed a rapid rate of influx up to 10 minutes, followed by a negative rate indicative of efflux

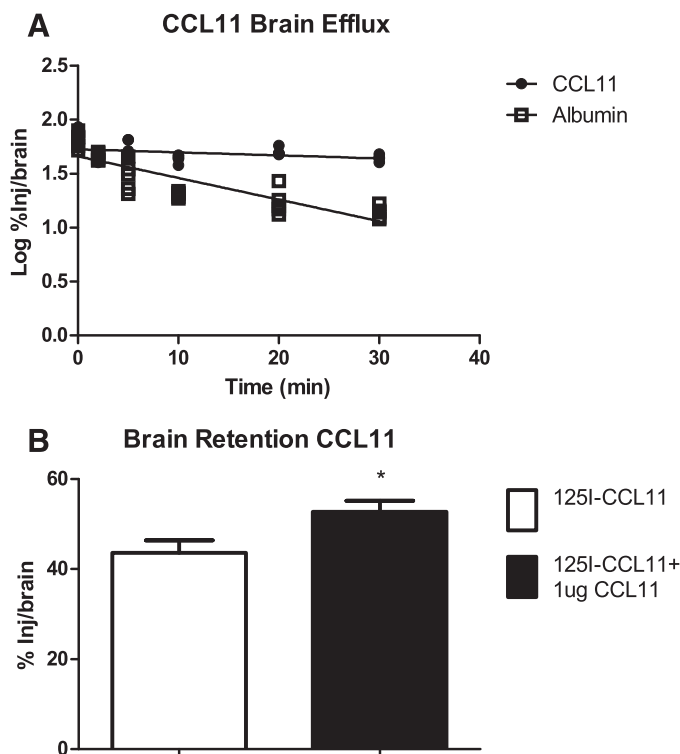


Fig. 10. Brain efflux of CCL11. Time curves for quantification of 125 I-CCL11 and 131 I-albumin efflux rates are shown in (A). Slopes of the lines for CCL11 and albumin were -0.002811 ± 0.001323 ($r^2 = 0.1388$, $df = 28$) and -0.02002 ± 0.002256 ($r^2 = 0.773$, $df = 28$), respectively. Both slopes were significantly non-zero. Brain retention of 125 I-CCL11 with or without coinjection of 1 μ g of unlabeled CCL11 was measured at 10 minutes postinjection intracerebroventricularly. (B) Data were analyzed by two-tailed unpaired t test, $n = 7$ or 8 per group. $*P < 0.05$.

TABLE 3
Regional uptake of CCL11

Region	Slow Phase			Fast Phase		
	K_i	r^2	df	K_i	r^2	df
OB	0.2187 ± 0.4460	0.03322	7	2.866 ± 0.7569*	0.642	8
ST	5.626 ± 2.092*	0.5082	7	-2.315 ± 1.860	0.1622	8
FC	0.5169 ± 0.2293	0.4207	7	1.929 ± 0.3344*	0.8061	8
HY	1.638 ± 0.8032	0.3728	7	2.574 ± 0.5896*	0.7044	8
HC	1.024 ± 0.5984	0.2949	7	2.169 ± 0.5910*	0.6275	8
TH	0.6207 ± 0.2951	0.3872	7	1.942 ± 0.3951*	0.7511	8
PC	0.6052 ± 0.2463*	0.4631	7	1.825 ± 0.3908*	0.7317	8
OC	0.7037 ± 0.2821*	0.4707	7	1.521 ± 0.6056*	0.4407	8
CB	1.108 ± 0.3609*	0.5741	7	2.047 ± 0.4222*	0.746	8
MB	0.4920 ± 0.2479	0.3601	7	2.101 ± 0.4123*	0.7645	8
PM	0.5431 ± 0.3811	0.2249	7	1.988 ± 0.3578*	0.7943	8

*Statistically significant uptake by brain.

between 10 and 30 minutes. This is supported by findings of significantly higher accumulation of CCL11 in striatum compared with all other brain regions by 10 minutes, but not after 30 minutes. Substantial accumulation of CCL11 in all brain regions tested may therefore be indicative of some physiologic function of CCL11 in the brain. Recently, it was shown that a quantitative trait locus in proximity to CCL11 associates with disease severity in a rat model of multiple sclerosis (MS). Upregulation of CCL11 in lymph nodes and the spinal cord after MS induction was a feature of MS resistance at this locus (Adzemovic et al., 2012). This study also showed that CCL11 and its receptor CCR3 are both expressed by neurons, and a previous study supports a neuroprotective function for CCR3, as mice lacking CCR3 have increased facial motor neuron loss after facial nerve axotomy (Wainwright et al., 2009). Therefore, CCL11 may have both beneficial and harmful effects in the CNS that depend on factors such as concentration, anatomic location, and pathophysiological context. Striatum was one region of the brain that showed dissimilar uptake kinetics compared with other regions. It is noteworthy that the SVZ is in close proximity to the striatum and would have been included in our gross dissections of this region. Therefore, it is presently unclear whether 1) the unique uptake kinetics observed in our gross dissections of striatum are actually specific for the SVZ, and 2) why rapid uptake followed by rapid efflux occurs in this region. Although vascular leakiness in the SVZ is one possible explanation for rapid uptake of CCL11, specificity of uptake was evident in our study, because albumin did not appreciably accumulate in this region compared with others. Of interest, unique characteristics of the circulation in the SVZ were recently reported (Culver et al., 2013); these included reduced blood flow and a hypoxic environment. These properties did not extend to striatum, which supports the possibility that unique CCL11 transport in this region is indeed reflective of the SVZ. Another remaining question is why similar uptake kinetics are not observed in the hippocampus, which contains the SGZ. Future studies are certainly necessary to elucidate the relationship of CCL11 transport across the BBB in the context of its effects on neurogenesis versus other functions in the CNS.

Finally, we addressed whether CCR3 at the BBB played a role in CCL11 influx. CCL11 has high selectivity for CCR3, and this receptor is expressed on endothelial cells of the BBB

(Edinger et al., 1997; Mukhtar et al., 2002). To specifically determine the effects of CCR3 inhibition at the BBB, we copperfused CCL11 with different concentrations of a pharmacologic inhibitor of CCR3. Compared with vehicle, coprefusion of the lowest dose of inhibitor (approximately 10× the IC₅₀) caused a slight but nonsignificant decrease in CCL11 uptake. The highest concentration of inhibitor potentiated CCL11 uptake, and this effect was not a result of BBB disruption. Two possibilities could explain our observations of increased CCL11 transport in the presence of high concentrations of CCR3 inhibitor. The first is that transporters versus receptors for CCL11 at the BBB are likely distinct entities. Therefore, binding of CCL11 to a receptor may partially compete with CCL11 binding to a transporter at the BBB. Potent inhibition of CCR3 and possibly other lower-affinity receptors of CCL11 due to off-target effects of the inhibitor would eliminate such competition, allowing for increased transport. The second possibility is that CCR3 mediates efflux, but not influx of CCL11. In this scenario, higher doses of inhibitor would be required to achieve effective concentrations in the CNS to inhibit efflux. Currently, evidence of polarized CCR3 localization that would support a role in efflux is not available. Despite these uncertainties surrounding the effects of the high dose of CCR3 inhibitor, our results clearly show that CCR3 is not the predominant influx transporter for CCL11.

Together, our results clearly demonstrate that CCL11 crosses the BBB. CCL11 transport into the brain occurs in regions outside the neurogenic niche. This indicates that the BBB could be an important regulatory interface for CCL11 levels in the brain, with potential effects on both adult neurogenesis and possibly other functions of CCL11 in the CNS that have yet to be defined. Increased circulating levels of CCL11 have been implicated in other diseases associated with aberrant CNS function, including Alzheimer disease (Leung et al., 2013), schizophrenia (Teixeira et al., 2008), and depression (Grassi-Oliveira et al., 2012). Elucidating roles for CCL11 function in the CNS may provide evidence for causal relationships between CCL11 elevations in blood and these diseases. Here, we show that the BBB expresses both influx and efflux transporters for CCL11. This suggests that the BBB has the capacity to regulate CCL11 levels in the brain and could therefore influence its functions in the CNS. Furthermore, pathophysiological changes in CCL11 transporters at the BBB may have important consequences in regard to

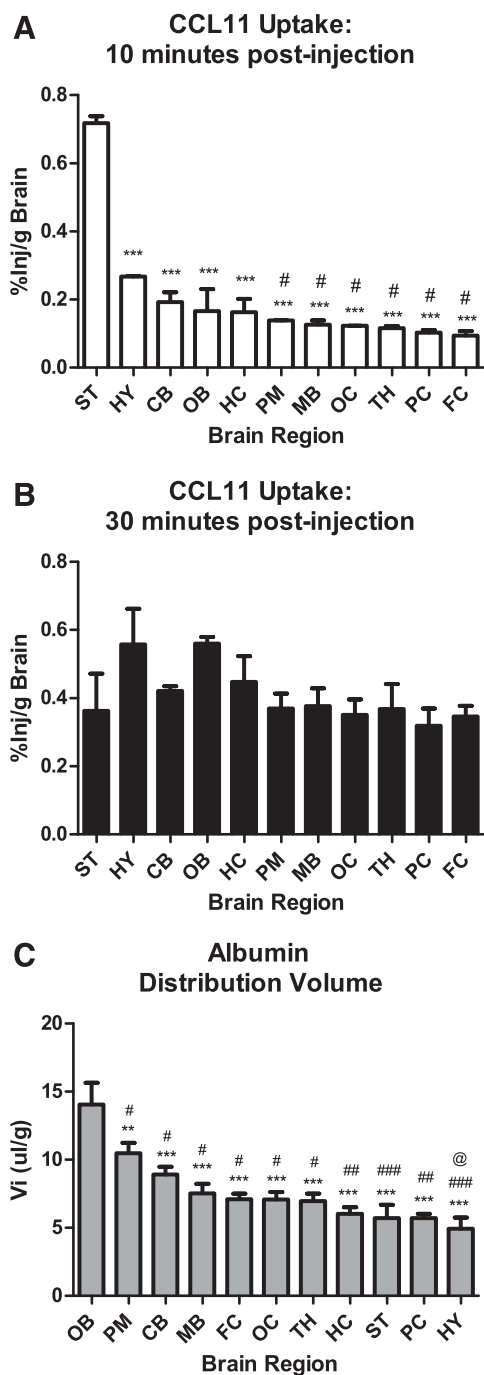


Fig. 11. Regional uptake of CCL11. Percent injection of ¹²⁵I-CCL11 per gram of brain tissue was determined at 10 (A) or 30 minutes (B) after intravenous injection. ¹³¹I-Albumin distribution volumes are shown in (C). Abbreviations are as follows: ST, striatum; HY, hypothalamus; CB, cerebellum; OB, olfactory bulb; HC, hippocampus; PM, pons-medulla; MB, midbrain; OC, occipital cortex; TH, thalamus; PC, parietal cortex; and FC, frontal cortex. Data were analyzed by one-way analysis of variance and Newman-Keuls multiple comparisons tests. For (A), ****P* < 0.001 versus striatum; #*P* < 0.05 versus hypothalamus; *n* = 2 per group. For (C), ***P* < 0.01; ****P* < 0.001 versus olfactory bulb; #*P* < 0.05; ##*P* < 0.01; ###*P* < 0.001 versus pons-medulla; @*P* < 0.05 versus cerebellum; and *df* = 14–15.

brain levels of CCL11 and the functions of CCL11 in the CNS. Therefore, the BBB is an important target to consider in future studies of pathophysiologic effects of the systemic milieu.

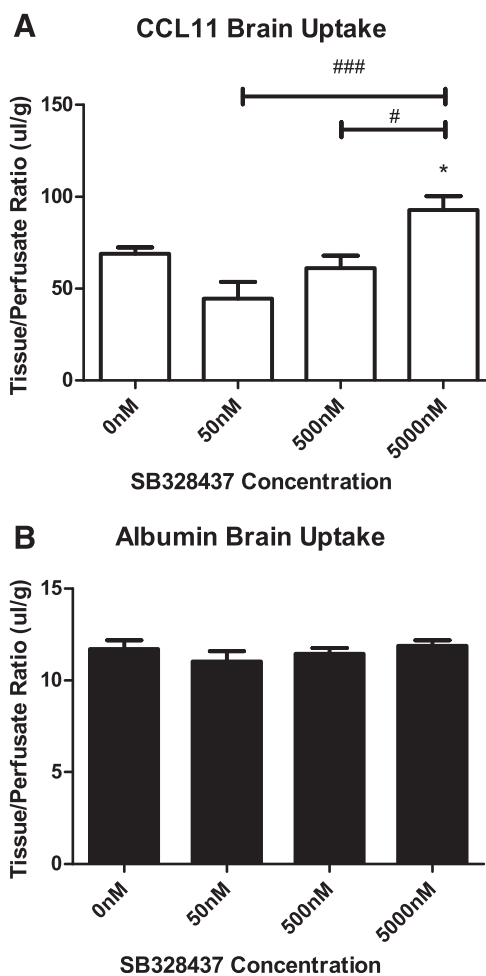


Fig. 12. Effects of CCR3 inhibitor on CCL11 uptake. In situ brain perfusions with ¹²⁵I-CCL11 (A) and ¹³¹I-albumin (B) were performed for 15 minutes in the presence or absence of the CCR3 inhibitor SB328437. Data were analyzed by one-way analysis of variance with Newman-Keuls multiple comparisons test. **P* < 0.05 versus 0 nm; #*P* < 0.05; ###*P* < 0.001 versus group indicated; *n* = 5 or 6 per group.

Authorship Contributions

Participated in research design: Erickson, Morofuji, Banks.
Conducted experiments: Erickson, Morofuji, Owen.
Performed data analysis: Erickson, Morofuji.
Wrote or contributed to the writing of the manuscript: Erickson, Morofuji, Owen, Banks.

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Address correspondence to: William A. Banks, Bldg. 1, Rm. 810A, 1660 Columbian Way, Seattle, WA 98108. E-mail: wabanks1@u.washington.edu