Title: Transport of the pro-inflammatory chemokines CCL2 (MCP-1) and CCL5 (RANTES) across the intact mouse blood-brain barrier is inhibited by heparin and eprodisate and increased with systemic inflammation.

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Running title: Regulation of CCL2 and CCL5 blood-brain barrier transport

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Number of pages: 52

Number of tables: 10

Number of figures: 14

Number of references: 77

Number of words in abstract: 225

Number of words in introduction: 745

Number of words in discussion: 1488

List of non-standard abbreviations: Blood-brain barrier (BBB), C-C Motif Chemokine Ligand 2 (CCL2), C-C Motif Chemokine Ligand 5 (CCL5), C-C Motif Chemokine Receptor 2 (CCR2), C-C Motif Chemokine Receptor 5 (CCR5), heparan sulfate (HS), heparan sulfate proteoglycan (HSPG) glycosaminoglycans (GAGs), lipopolysaccharide (LPS)
Abstract: One important function of the vascular blood-brain barrier (BBB) is to facilitate neuroimmune communication. The BBB fulfills this function, in part, through its ability to transport cytokines and chemokines. CCL2 (MCP-1) and CCL5 (RANTES) are pro-inflammatory chemokines that mediate neuroimmune responses to acute insults, and aspects of brain injury and neurodegenerative diseases; however, a blood-to-brain transport system has not been evaluated for either chemokine in vivo. Therefore, we determined whether CCL2 and CCL5 in blood can cross the intact BBB and enter the brain. Using CD-1 mice, we found that $^{125}$I-labeled CCL2 and CCL5 crossed the BBB, and entered the brain parenchyma. We next aimed to identify the mechanisms of $^{125}$I-CCL2 and $^{125}$I-CCL5 transport in an in-situ brain perfusion model. We found that both heparin and eprodisate inhibited brain uptake of $^{125}$I-CCL2 and $^{125}$I-CCL5 in situ, whereas antagonists of their receptors, CCR2 or CCR5 respectively, did not, suggesting that heparan sulfates at the endothelial surface mediate BBB transport. Finally, we showed that CCL2 and CCL5 transport across the BBB increased following a single injection of 0.3mg/kg lipopolysaccharide. These data demonstrate that CCL2 and CCL5 in the brain can derive, in part, from the circulation, especially during systemic inflammation. Further, binding to the BBB-associated heparan sulfate is a mechanism by which both chemokines can cross the intact BBB, highlighting a novel therapeutic target for treating neuroinflammation.

Significance statement: Our work demonstrates that CCL2 and CCL5 can cross the intact BBB, and that transport is robustly increased during inflammation. These data suggest that circulating CCL2 and CCL5 can contribute to brain levels of each chemokine. We further show that the transport of both chemokines is inhibited by heparin and eprodisate, suggesting that...
CCL2/CCL5-heparan sulfate interactions could be therapeutically targeted to limit accumulation of these chemokines in the brain.

**Introduction:**

C-C Motif Chemokine Ligand 2 (CCL2/ MCP-1) and C-C Motif Chemokine Ligand 5 (CCL5/RANTES) are chemokines that are important modulators of the neuroinflammatory response (Semple et al., 2010b; Williams et al., 2014; Pittaluga, 2017). Both chemokines mediate leukocyte recruitment and are upregulated in response to inflammation or injury (Huang et al., 2000; Semple et al., 2010b). CCL2 and CCL5 signal predominantly through the G-protein coupled receptors CCR2 and CCR5, respectively (Huang et al., 2000; Appay and Rowland-Jones, 2001; Semple et al., 2010b). However, heparan sulfate (HS) binding also regulates CCL2 and CCL5 functions by facilitating the formation of haptotactic gradients that direct leukocyte movements, and by increasing chemokine residence time on the endothelium which promotes ligand-receptor interactions (Appay and Rowland-Jones, 2001; Proudfoot et al., 2001; Lau et al., 2004; Graham et al., 2019). HS is highly expressed on the luminal (blood-facing) surface of endothelial cells as a major constituent of the glycocalyx, a carbohydrate-rich interface that facilitates interactions between endothelial cells and circulating substances (Reitsma et al., 2007). The HS in glycocalyx is typically covalently linked to cell-associated proteoglycan core proteins (heparan sulfate proteoglycans, HSPGs) such as syndecans and glypicans (Christianson and Belting, 2014; Oshima et al., 2021). HSPGs can also facilitate the transcytosis of substances across endothelial cells (Christianson and Belting, 2014). Many chemokines, including CCL2 and CCL5, bind HS (Proudfoot et al., 2001; Lau et al., 2004; Bao et al., 2010).
The vascular blood-brain barrier (BBB) is comprised of highly specialized endothelial cells that regulate the passage of substances into and out of the brain (Daneman and Prat, 2015). Brain endothelial cells control the passage of circulating substances into the brain by preventing paracellular (between-cells) and transcellular (across cells) leakage. Specialized tight junctions that form between brain endothelial cell contacts inhibit paracellular leakage (Berndt et al., 2019), and transcellular leakage is largely suppressed through the inhibition of macropinocytosis by Major Facilitator Superfamily Domain Containing 2A (Ben-Zvi et al., 2014; Andreone et al., 2017). Conversely, brain endothelial cells selectively facilitate the passage of substances into and out of the brain through their expression of transporters, which are essential for delivery of nutritive and trophic substances that support CNS functions (Abbott et al., 2010). The BBB both contributes to the immune-privileged status of the CNS (Pachter et al., 2003) and is also an important CNS-immune interface, participating in several neuroimmune axes (Erickson and Banks, 2018).

One immune interface function of the BBB is its ability to transport cytokines and chemokines between the blood and brain compartments. Blood-to-brain (influx) and brain-to-blood (efflux) transport systems have been described for many pro-inflammatory cytokines and chemokines (Banks, 2015). This includes efflux of CCL2 and CCL5 in vitro which, assuming it also occurs in vivo, may contribute to leukocyte recruitment from the circulation (Ge et al., 2008; Minten et al., 2014)(Middleton et al., 2002; Rot, 2003). However, whether CCL2 and CCL5 that originate in the circulation could also interact with the blood-facing (luminal) side of the BBB has not yet been investigated.
In this study, we first aimed to determine whether CCL2 and CCL5 could cross the intact mouse BBB in the blood-to-brain direction via influx. Using highly sensitive radiochemical-based assays, we showed in vivo that $^{125}$I-CCL2 and $^{125}$I-CCL5 both have influx transport systems, and are transported across the BBB into brain parenchyma. We then aimed to identify the transport mechanism using an in-situ brain perfusion approach. CCR2 and CCR5 are the predominant receptors for CCL2 and CCL5, respectively, that are expressed on brain endothelial cells (Subileau et al., 2009). CCR2 and CCR5 receptor antagonists did not significantly affect the rate of brain uptake for its respective chemokine. In contrast, both heparin (a highly sulfated form of heparan sulfate) and eprodisate, an HS mimetic that has been clinically evaluated for the treatment of renal amyloidosis (Kisilevsky et al., 1995; Dember et al., 2007), significantly inhibited CCL2 and CCL5 influx. Finally, we determined whether CCL2 and CCL5 transport across the BBB and into peripheral tissues was altered by systemic inflammation, which we induced using bacterial lipopolysaccharide (LPS) at a low dose that causes sickness behaviors and neuroinflammation without BBB leakage (Godbout et al., 2005; Banks et al., 2015). LPS induced substantial increases in the brain influx of both CCL2 and CCL5. Our results suggest that circulating CCL2 and CCL5 contribute to brain levels, especially in the context of systemic inflammation. HS mediates brain uptake of circulating CCL2 and CCL5, highlighting a novel mechanism for transport of these chemokines across the BBB.

**Materials and Methods:**

**Animal Subjects**

CD-1 male and female 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 reached 8 to 10 weeks of age. All studies were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee of the Veterans Administration Medical Center. Blinding was not used in our study designs due to feasibility and the need-to-know experimental groups for appropriately carrying out the studies and analysis. The studies were not randomized, but treatment and control groups were balanced on each study day and experiments carried out over multiple study days to mitigate the influence of artifacts and ensure reproducibility.

**Lipopolysaccharide treatments**

Bacterial lipopolysaccharide from *Salmonella enterica* serotype *typhimurium* (LPS, L6511) was purchased from Sigma-Aldrich (St. Louis, MO). LPS was prepared for injection by dissolving in sterile normal saline at a concentration of 0.1mg/ml, and filter sterilizing using a 0.22µm PES filter on the day of use. Mice were weighed and injected intraperitoneally (i.p.) with 0.3mg/kg LPS, or sterile normal saline vehicle once in the morning between the hours of 7:00 and 9:00. Studies were carried out 27-29 hours post-injection.

**Iodination of CCL2 and CCL5 and Iodine or Technetium Labeling of Albumin**

Murine recombinant carrier-free CCL2 (479-JE-050/CF) and murine recombinant carrier-free CCL5 (478-MR-025/CF) were purchased from R&D Systems (Minneapolis, MN). Lycopersicon esculentum lectin (LEL) was purchased from Vector Laboratories (Newark, CA). Bovine serum albumin (BSA, 17030-100G) was purchased from Sigma-Aldrich (St. Louis, MO). CCL2, CCL5, and LEL were labeled with $^{125}$I via the chloramine-T method as was described
previously (Hunter and Greenwood, 1962). 5 µg of chemokine or 10ug LEL was incubated with 0.5mCi $^{125}$I (carrier free, Perkin Elmer) and 10 µg of chloramine-T (Sigma Aldrich) for 1 minute in 0.25 M phosphate buffer. The reaction was terminated by an addition of 100 µg of sodium metabisulfite (Sigma Aldrich), followed by purification on a G-10 Sephadex column. Specific activities were estimated to be 31.1 Ci/g for CCL5 and 41.42 Ci/g for CCL2. BSA was labeled with $^{131}$I (carrier free, GE Healthcare) using the chloramine T method, as described above for chemokines, or with $^{99m}$Tc using the stannous tartrate method as was described previously (Wang et al., 2007). BSA labeled with either isotope behaves indistinguishably, and later experiments with LPS were conducted with $^{131}$I-labeled BSA which remains stable for up to two weeks post-labeling vs. $^{99m}$Tc-BSA which requires labeling fresh each day. For $^{99m}$Tc labeling, 1 mg albumin was labeled in 500 µl of deionized water with 120-250 µg tin(II) tartrate (S4895) (Sigma-Aldrich, St. Louis, MO) at a pH of 2.5-3.3 with 1 mCi $^{99m}$Tc. The mixture was allowed to incubate for 20 minutes before purification on a Sephadex G-10 column to yield labeled albumin ($^{99m}$Tc-Alb) with an estimated specific activity of 103.6 Ci/g for albumin. Protein labeling by iodine and technetium isotopes was characterized by precipitation with 15% trichloroacetic acid (TCA); in the case of LEL, a saturating concentration of sodium chloride was added to the TCA to facilitate precipitation of the glycoprotein. Greater than 97% radioactivity in the precipitated protein fraction was consistently observed for CCL5 and CCL2, and greater than 90% and 98% radioactivity in the precipitated fraction was consistently observed for $^{99m}$Tc-Alb and $^{131}$I-Alb, respectively. $^{125}$I-LEL consistently had greater than 90% of the total radioactivity in the acid-brine precipitated pellet. Labeled chemokines were further verified by SDS-PAGE, described below.

**Verification of Iodinated Chemokines by SDS-PAGE**
To establish the apparent molecular weight of the unlabeled proteins, 1 µg of CCL2 or CCL5 was run on a pre-cast 4-20% bis-tris PAGE gel (M42010) (GenScript, Piscataway, NJ) in reducing conditions as per the manufacturer’s instructions. 5 µl of SeeBlue plus2 protein ladder (LC5925) (Thermo Fisher, Waltham, MA) was used as a size reference. Gels were then stained with SYPRO Ruby Protein Gel Stain (S12001) (Thermo Fisher, Waltham, MA) as per the manufacturer’s instructions. Imaging was performed on an Amersham ImageQuant 800 Biomolecular Imager (29399481) (General Electric, Boston, MA) on the UV fluorescence setting with a single exposure.

Following iodination, 2(10^5) counts per minute (CPM) of ^125I-CCL2 or ^125I-CCL5 were run on a pre-cast bis-tris PAGE gel as described for unlabeled chemokines. Once the gel run was completed the gel was washed twice in deionized water, then incubated in Invitrogen Gel-Dry Drying solution (LC4025) (Thermo Fisher, Waltham, MA) for 15 minutes. The gel was then dried out overnight as per the manufacturer’s instructions. The gel was exposed to autoradiography film (NC9252739) (Thermo Fisher, Waltham, MA) in a light-proof exposure cassette for 24 hours and developed on a Mini-Medical/90 X-ray Film Processor (9992305000) (AFP imaging, Elmsford, NY).

**In vivo measurement of BBB transport**

To determine the kinetics of ^125I-CCL2 and CCL5 brain uptake across the BBB, mice were anesthetized with intraperitoneal urethane and given an intravenous co-injection of 3(10^5) CPM ^125I-chemokine (CCL2 or CCL5) and 10^6 CPM ^99mTc-Alb or 3(10^6) CPM ^131I-Alb (to quantify vascular space) into the left jugular vein. After a circulation time from 1 to 30 minutes, serum, whole brains, and in some studies, peripheral organs were collected. In some
experiments, mice were perfused with lactated Ringer’s solution at a rate of 15 ml/min to clear blood from the vascular space and to wash away radiotracers that were weakly bound to the vascular lumen. In some experiments, the brains were dissected into 11 regions (Erickson et al., 2022). The CPM in brain and serum were measured in a Wizard2 automatic gamma counter (PerkinElmer, Waltham, MA). The amount of radioactivity in the brain and serum was expressed as the percent of injected CPM/g of brain tissue or per µl of serum, respectively (%Inj/g or %Inj/µl). The brain/serum ratios were then calculated by dividing %Inj/g by %Inj/µl to give units of µl/g. Multiple-time regression analysis (Blasberg et al., 1983; Kastin et al., 2001) was used to determine the rates of chemokine uptake into the brain. This method plots the brain/serum ratios of the compound of interest against a corrected time parameter, exposure time, which is derived from the equation:

\[
\text{Exposure time} = \left[ \int_0^t C_p(\tau) d\tau \right] / C_{pt}
\]

Where \( t \) is the time between i.v. injection and blood draw, \( C_p \) is the level of radioactivity in serum (expressed as %Inj/ml), \( C_{pt} \) is the level of radioactivity in serum at time \( t \), and \( \tau \) is the dummy variable for time. Exposure time corrects for the clearance of the compound of interest from blood, and so the slope of the linear portion of the brain/serum ratio vs. exposure time relation measures the unidirectional influx rate (\( K_i \), in units of µl/g/min), and the Y-intercept measures \( V_i \) (in units of µl/g), which is the vascular space and initial luminal binding at \( t=0 \). 99mTc-Alb is a marker of the vascular space, and any non-specific leakage into tissues that occurs. For 125I-CCL2 and -CCL5 uptake calculations, 99mTc-Alb or 131I-Alb brain/serum ratios were subtracted from the chemokine brain/serum ratios, yielding a ‘delta’ value. The delta
values thus correct for unbound chemokines in the residual blood that occupies the vessel lumen and non-specific leakage into the brain.

**In situ measurement of BBB transport**

In situ brain perfusions were performed as described previously (Banks et al., 2000). Mice were anesthetized with intraperitoneal urethane, and the thorax was opened to expose the heart. 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 CaCl2, 2.1 g/l NaHCO3, 0.16 g/l KH2PO4, 0.17 g/l anhydrous MgCl2, and 0.99 g/l D-glucose, with 10 g/l bovine serum albumin added on the day of perfusion) containing 3(10⁴) ¹²⁵I-chemokine (CCL2 or CCL5) or ¹²⁵I-LEL, 6(10⁴) cpm/ml ⁹⁹mTc-Alb, or 1.5(10⁴) ¹³¹I-Alb was infused at a rate of 2 ml/min for 1–10 minutes. After perfusion, the mouse was decapitated and the brain was removed and counted in a gamma counter. The perfusion fluid was also counted. Brain/perfusate ratios were calculated by dividing the CPM/g of brain tissue by the CPM/ul of perfusion fluid to yield units of microliters per gram. Because there is no clearance of chemokine or albumin from the perfusate, brain/perfusate ratios were plotted against clock time to determine the Ki and Vi. In some experiments, freshly prepared CCR2 inhibitor RS504393 (SML0711) (Sigma-Aldrich, St. Louis, MO) or CCR5 inhibitor Maraviroc (PZ0002) (Sigma-Aldrich, St. Louis, MO) or vehicle (dimethylsulfoxide) were included. Perfusions in these experiments lasted 1-10 minutes and the final inhibitor concentration in the perfusate was 10μM for both inhibitors; the final concentration of DMSO was 0.2%. In other experiments, 1,3-propanedisulfonic acid disodium salt (eprodisate) (P8579-25G) (Sigma-Aldrich, St. Louis, MO) or heparin sodium salt (heparin)
(H3393) (Sigma-Aldrich, St. Louis, MO) were added to the perfusate in concentrations of 50 mM and 20 U/ml, respectively. In further, separate experiments, differential doses of eprodisate (0, 0.5, 5.0 and 50 mM) were also administered at a single time point of 5 minutes to generate a dose-response curve for both CCL5 and CCL2. In all studies, we verified that the BBB remained intact by co-perfusing $^{99m}$Tc-Alb. The perfused brain remains viable for approximately 7 hours (Krieglstein et al., 1972a), and the BBB remains intact until approximately 12 hours after death (Broman et al., 1950; Grontoft, 1954).

**Capillary Depletion in Mice**

To determine whether CCL5 and CCL2 completely crossed the BBB into brain parenchyma, we performed capillary depletion as adapted to mice (Triguero et al., 1990; Gutierrez et al., 1994). This method causes an enrichment of capillaries which was originally confirmed by measuring the activity of the endothelial-specific $\gamma$-glutamyl transpeptidase enzyme. Following capillary depletion, the parenchymal fraction had 1.91% of the enzymatic activity that was present in the capillary fraction, indicating capillary enrichment (Gutierrez et al., 1993). We have consistently found similar enrichment as well by comparing the capillary content of the pellet vs. parenchymal fraction by light microscopy. Mice anesthetized with intraperitoneal urethane received an intravenous injection of $3 \times 10^5$ CPM of $^{125i}$-chemokine (CCL2 or CCL5) and $10^6$ CPM of $^{99m}$Tc-Alb. 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 was washed free of blood by perfusing 20 ml of lactated Ringer’s solution through the left ventricle of the heart. The brain was removed, weighed, placed in 0.8 ml of ice-cold physiologic buffer (10 mM HEPES, 141
mM NaCl, 4 mM KCl, 2.8 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, and 10 mM D-glucose adjusted to pH 7.4) and rapidly counted in a gamma counter to quantify the total radioactivity in brain. The brain was then homogenized with 10 strokes of a glass homogenizer, and an equal volume of 40% dextran solution was added to the homogenate, which was thoroughly mixed with 3 strokes of a glass homogenizer. 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 levels of ¹²⁵I and ⁹⁹mTc were determined in a gamma counter along with serum. The tissue/serum ratios of the capillary and parenchymal fractions were calculated by determining the proportion of total recovered CPM in the respective fraction, and then multiplying the proportion by the tissue/serum ratio calculated for whole brain.

Acid Precipitation of ¹²⁵I-CCL2, ¹²⁵I-CCL5, and ⁹⁹mTc-albumin from blood and brain tissues

To determine whether the ¹²⁵I-CCL2 and ¹²⁵I-CCL5 that entered the brain was intact, we performed acid precipitations of serum and brain homogenates. Serum was collected following intravenous injection of 3(10⁵) CPM ¹²⁵I-chemokine (CCL2 or CCL5) into the left jugular vein. After a circulation time of 5, 10, or 30 minutes, blood was collected from the abdominal aorta, placed on ice for 30 minutes, and then centrifuged at 3500g for 10 minutes. Processing controls were also included where 10⁵ CPM of ¹²⁵I-chemokine (CCL2 or CCL5) was added to the blood collection tube, blood was collected from a non-radioactive mouse, and processed along with the test samples. The processing controls are used to estimate the percent degradation that results ex vivo due to tissue processing. 10 µl of serum was then added to 490µl 1% BSA in lactated
Ringer’s, and 500 µl of 30% TCA was added and mixed. Samples were centrifuged at 4500g for 10 minutes at 4°C, and then the pellets and the supernatants were counted in a gamma counter. Brains were collected following in situ brain perfusion of $3 \times 10^4$ cpm/ml $^{125}$I-chemokine (CCL2 or CCL5) for 10 minutes, and homogenized in 3mls of 1% BSA in lactated Ringer’s solution plus cOmplete Mini protease inhibitor cocktail (11836153001) (Sigma Aldrich, St. Louis, MO). Processing controls were also included where $10^5$ CPM of $^{125}$I-chemokine (CCL2 or CCL5) was added to the brain collection tube, non-radioactive brains were collected and processed along with the test samples. The homogenates were centrifuged at 4500g at 4°C for 10 minutes and then 500µl of supernatant was mixed with 500 µl 30% TCA and centrifuged at 4500g at 4°C for 10 minutes. The pellets and supernatants were then counted in a gamma counter.

Statistics

Prism 8.0 was used for all statistical calculations (GraphPad Software, Inc. San Diego, CA). Linear regression analysis was used to calculate slopes and intercepts, and the standard errors were reported for the slope and y intercepts. Differences in the slopes and intercepts of two lines were evaluated by ANCOVA. Comparison of means group trends was performed using one-way or two-way ANOVA with Dunnett’s or Sidak’s multiple-comparisons testing, respectively. Comparison of clearance curves was done using the extra sum-of squares F-test, and the global fits of the curves were compared.

Results

Characterization of BBB influx of CCL2 and CCL5
After verifying that $^{125}$I-CCL2 and $^{125}$I-CCL5 remained intact post-labeling (Figure 1), we determined whether intravenously injected $^{125}$I-CCL2 and $^{125}$I-CCL5 could cross the intact mouse BBB using multiple-time regression analysis. In all experiments, $^{99m}$Tc-Alb was co-injected to quantify the vascular space, and brain/serum ratios for $^{99m}$Tc-Alb were subtracted from brain/serum ratios of each chemokine. $^{125}$I-CCL2 (Figure 2A) and $^{125}$I-CCL5 (Figure 3A) were cleared rapidly from blood with half-lives of 0.7921 and 0.6583 minutes, respectively. The clearance of $^{99m}$Tc-Alb in each experiment is shown for comparison (Figure 2B and 3B). Brain uptake curves in Figure 2C-D/Table 1 and Figure 3C-D/Table 2 showed that $^{125}$I-CCL2 and $^{125}$I-CCL5 had higher Vi’s vs. $^{99m}$Tc-Alb in both the washout and no washout conditions, indicating vascular binding. To distinguish weak binding of the vascular surface from endothelial/brain uptake, the brain vasculature was washed out by rapid perfusion, which clears about 80-90% of the vascular space, as shown by the reduction in $^{99m}$Tc-Alb Vi with washout (Tables 1 and 2). Vascular washout post-$^{125}$I-CCL2 injection significantly lowered the Vi vs. no washout (Table 1), and also resulted in a measurable slope, indicating that there was brain uptake. The comparison of washout vs. no washout for CCL2 indicated that brain/serum ratios in the no washout group are attributable to weak vascular binding at early timepoints, but that BBB transport predominates at later timepoints. Vascular washout post-$^{125}$I-CCL5 injection also significantly lowered the Vi vs. no washout (Table 2), and resulted in a significantly non-zero slope, indicating brain uptake as well. The comparison of washout vs. no washout for CCL5 indicated that brain/serum ratios are attributable to weak binding and brain uptake for the duration of the time course. Notably, there were apparent differences in CCL2 vs. CCL5 brain uptake patterns.

Saturability of CCL2 and CCL5 BBB transport
The saturability of $^{125}$I-CCL2 and $^{125}$I-CCL5 vascular binding and brain uptake was next determined by co-injecting with or without 1µg of unlabeled CCL2 or CCL5. In comparison, about 2-4ng of $^{125}$I-CCL2 or $^{125}$I-CCL5 was injected per mouse. Injection of 1µg CCL2 reduced the clearance of $^{125}$I-CCL2 from blood (Figure 4A), as indicated by a statistical difference in the curve fits ($F= 8.218$ (3, 30), $p=0.0004$). The half-life of clearance went from 1.005 minutes when no unlabeled CCL2 was included in the injection to 2.801 minutes when 1µg CCL2 was co-injected. In contrast, injection of 1ug CCL5 did not significantly change the curve fit for $^{125}$I-CCL5 ($F= 0.5184$ (3, 8), $p= 0.6812$). The half-life went from 1.390 minutes to 1.550 minutes when 1ug unlabeled CCL5 was injected (Figure 4B). This difference in the apparent saturability of clearance could indicate that CCL2 is partly cleared from blood by a receptor-dependent mechanism. Multiple-time regression analysis was performed in mice that did not have vascular washout to capture the effects on both binding and transport. Injection of unlabeled CCL2 or CCL5 had no significant effects on the Ki or Vi of $^{125}$I-CCL2 or $^{125}$I-CCL5, respectively (Figure 4C and D, Table 2). 1µg of unlabeled CCL2 or CCL5 also had no significant effect on $^{99m}$Tc-Alb Ki or Vi (data not shown), indicating that the BBB remained intact for the duration of study.

**Capillary depletion studies**

We next determined whether $^{125}$I-CCL2 and $^{125}$I-CCL5 were transported completely across the BBB into brain parenchyma using the capillary depletion method. In this design, mice were injected with $^{125}$I-CCL2 or $^{125}$I-CCL5 together with $^{99m}$Tc-Alb. After a 30 minute circulation time, the vasculature was washed out to remove any loosely-bound material, and then the amount of radioactivity that partitioned into the capillary vs. parenchymal fractions was determined. Figure 5 shows the capillary vs. parenchymal uptake for $^{125}$I-CCL2 and $^{125}$I-CCL5.
compared to $^{99m}$Tc-Alb for reference. Two-way ANOVA identified significant main effects for injected material ($^{125}$I-CCL2 vs. $^{99m}$Tc-Alb, $F(1, 12) = 188.0, p<0.001$ and $^{125}$I-CCL5 vs. $^{99m}$Tc-Alb, $F(1, 12) = 226.6, p<0.001$), and brain compartment (capillary vs. parenchyma, $^{125}$I-CCL2, $F(1, 12) = 59.93, p<0.001$ and $^{125}$I-CCL5 $F(1, 12) = 105.6$), as well as an interaction ($^{125}$I-CCL2, $F(1, 12) = 55.00, p<0.001$ and $^{125}$I-CCL5 $F(1, 12) = 99.29$). There was significantly more $^{125}$I-CCL2 and $^{125}$I-CCL5 present in the brain parenchymal fractions vs. capillary fractions, demonstrating that the majority of each chemokine completely crosses the BBB into the brain parenchyma. Significant vascular binding/internalization was also apparent, as evidenced by the significantly higher brain/serum ratios of $^{125}$I-CCL2 or $^{125}$I CCL5 vs. $^{99m}$Tc-Alb in the capillary fraction.

*Acid precipitation to verify $^{125}$I-CCL2 and $^{125}$I-CCL5 integrity in blood and brain*

To verify that the $^{125}$I measurements in blood and brain reflect that of intact chemokines, we conducted acid precipitations. Following intravenous injections, arterial blood was collected after a circulation time of 5, 10, or 30 minutes. We found that the circulating $^{125}$I-CCL2 remained greater than 90% intact for up to 10 minutes, and that $^{125}$I-CCL5 was degraded more rapidly after about 5 minutes (Table 6). Although we have found that iodine degradation products contribute minimally to brain uptake calculations using multiple-time regression analysis, we conducted acid precipitations of brains perfused with $^{125}$I-CCL2 and $^{125}$I-CCL5 for 10 minutes to verify that their brain uptake was intact in the absence of serum degradation. We found that both chemokines remained completely intact in the brain in the perfusion model when compared to processing controls (Table 6). The slightly lower acid precipitation of each
chemokine in the processing controls may be attributed to residual blood in the processing control brains.

**Effects of chemokine receptor inhibitors, heparin, and eprodisate on the BBB transport of $^{125}$I-CCL2 and $^{125}$I-CCL5 in situ.**

We next characterized the mechanism of $^{125}$I-CCL2 and $^{125}$I-CCL5 transport across the BBB using an in situ brain perfusion method, which eliminates possible confounds of inhibitor clearance and interactions with peripheral tissues and blood. Perfused brains remain viable for approximately 7 hours (Krieglstein et al., 1972b), and the BBB remains intact as evidenced by the albumin vascular space remaining constant over time in this study and others (Banks et al., 2000). We first tested whether CCR2 and CCR5, the receptors for CCL2 and CCL5, respectively, were required for transport by co-perfusing $^{125}$I CCL2 or $^{125}$I CCL5 with a competitive antagonist for their respective receptor. We found that neither RS504393 (the CCR2 antagonist) nor Maraviroc (the CCR5 antagonist) significantly affected the brain uptake of $^{125}$I-CCL2 or $^{125}$I-CCL5 (Figure 6 and Table 4), suggesting that CCR2 and CCR5 are not transporters for these chemokines. We next evaluated the effects of heparin and a heparan sulfate mimetic, eprodisate, on BBB transport of $^{125}$I-CCL2 or $^{125}$I-CCL5 (Figure 7 and Table 5). Both heparin and eprodisate significantly lowered the rate of BBB transport (Ki) of $^{125}$I-CCL2, and eprodisate significantly lowered the transport rate of $^{125}$I-CCL5. Heparin lowered the Y-intercept (Vi), indicating that there was reduced initial vascular binding, and there was a trend towards a decreased slope ($p=0.0586$). To confirm that the suppressive effects of heparan on chemokine transport were selective, we conducted a study using tomato (*Lycopersicon esculentum*) lectin (LEL) labeled with $^{125}$I. LEL binds N-acetylglucosamine residues, which are a component of HS
chains, but a recent study showed that LEL preferably binds keratan sulfate with minimal binding to HS (Itakura et al., 2017). Consistent with these findings, we found that HS does not inhibit the brain uptake of $^{125}$I-LEL, supporting the specificity of the assay (Figure 8, Table 7). Because of the novel findings that eprodisate inhibits both CCL2 and CCL5 transport across the BBB, we further evaluated the concentrations that are required to inhibit transport. We found that only the 50 mM concentration of eprodisate significantly lowered the mean $^{125}$I-CCL2 brain/perfusate ratio vs. vehicle (Figure 9A), however there was a significant linear trend of the doses ($F(1, 28) = 22.12, p<0.001$). $^{125}$I-CCL5 brain/perfusate ratios were significantly lowered at all concentrations of eprodisate (Figure 9B), and there was a significant linear trend of the doses ($F(1, 30) = 20.15, p<0.001$). These data indicate that eprodisate is slightly more effective at inhibiting CCL5 transport across the BBB vs. CCL2.

Effect of LPS on CCL2 and CCL5 transport into the brain and peripheral tissues

CCL2 and CCL5 are pro-inflammatory chemokines that are elevated in blood and brain following acute systemic inflammatory insults (Thompson et al., 2008; Erickson and Banks, 2011). In addition to characterizing their transport across the BBB in the basal state, we wanted to determine whether CCL2 or CCL5 transport was altered with systemic inflammation. To test this, we injected male and female CD-1 mice with a single 0.3mg/kg dose of LPS, which causes moderate neuro- and systemic inflammation and sickness behaviors that resolve after about 72 hours in young mice (Godbout et al., 2008). We then used in situ brain perfusion to assess CCL2 and CCL5 brain uptake 27-29 hours post LPS injection. All mice were studied with brain perfusion for 10 minutes. $^{131}$I-albumin was used as a vascular space marker for all LPS studies, as it is interchangeable with $^{99m}$Tc-albumin and does not require daily labeling due to its longer
half-life. Both CCL2 and CCL5 brain uptake were robustly increased by LPS (Figure 10). For CCL2, we compared males and females and found that there was no significant effect of sex on brain uptake. LPS treatment accounted for 88.76% of the total variation (F (1, 20) = 166.6, p<0.001), and increased brain uptake by about 8-13-fold depending on sex. The albumin vascular space was slightly but significantly increased in male mice, and trended towards an increase in females. Our group has found that this dose of LPS does not cause BBB leakage (Banks et al., 2015), suggesting that the increased albumin was due to other factors such as vasodilation. Albumin vascular space was subtracted from the CCL2 and CCL5 brain uptake data so that the delta values reflect only the brain uptake of chemokines. The brain uptake of CCL5 was also significantly increased in female CD-1 mice treated with 0.3mg/kg LPS by about 36-fold. These data support that CCL2 and CCL5 transport across the BBB is strongly increased during systemic inflammation.

We next determined whether LPS altered the binding and uptake of CCL2 and CCL5 into the brain and peripheral organs including lungs, liver, kidney, and spleen in vivo using multiple-time regression analysis. These organs were specifically chosen to reflect a tissue-specific heterogeneity in the vasculature and immune functions for comparison. In this cohort, equal numbers of male and female CD-1 mice were used, and data from both sexes was combined for analysis and coded in the figures to reflect males (filled shapes) and females (open shapes). We did not observe sex-dependent differences in our measurements, except for serum clearance due to different body sizes of males and females affecting the initial distribution volumes of injected proteins. Therefore, clearance was compared within sex for albumin, CCL2, and CCL5 and no significant differences in the clearance curves were found with LPS treatment (Figures 11-13). We first report the uptake and distribution of albumin in each organ (Figure 11 and Table 8).
LPS significantly increased the albumin vascular space in the brain, liver, and kidney and decreased vascular space in the lung. A significant trend of time-dependent uptake was observed for kidney in the saline-treated group only, which is consistent with receptor-dependent uptake of albumin in the proximal tubule cells that decreases with LPS (Schreiber et al., 2012). The spleen also showed significant time-dependent uptake of albumin in both LPS and saline treated mice. To calculate the vascular space and time-dependent uptake for CCL2 and CCL5, the tissue/serum ratios of albumin were subtracted from those of CCL2 and CCL5. Figure 12 and Table 9 show that there was not a time-dependent uptake of CCL2 into the brain in either the saline or LPS-treated condition, but that the vascular space significantly increased with LPS, suggestive of increased weak binding and brain uptake, as shown in Figure 2. Significantly non-zero Ki, which indicates CCL2 organ uptake, was observed for lung, liver, kidney, and spleen although LPS did not significantly alter the rate of uptake for these organs. The liver showed a higher CCL2 vascular space with LPS, which could reflect increased binding to the vasculature. For CCL5, both lung and liver showed a non-linear phase of uptake after about 5 minutes of circulation, and so only the linear portion of the curve was used for the rate of tissue uptake calculations, although all data points are shown. A positive slope of the linear phase of uptake (Ki) was noted for lung, liver, kidney, and spleen in both the saline and LPS groups (Figure 13 and Table 10), indicating that peripheral organs contribute to uptake of $^{125}$I-CCL5 from blood. The lungs showed the fastest rate of uptake. LPS increased the rate of $^{125}$I-CCL5 uptake in the liver, but not in other organs. LPS significantly increased the vascular binding (Vi) of $^{125}$I-CCL5 in the brain and kidney without significantly affecting the rate of organ uptake (Ki) (Figure 12, Table 9). We note that these experiments did not include vascular washout, as we wanted to capture both binding and uptake of these chemokines into organs. We did not repeat these
studies with vascular washout as we did for the brain in Figures 2 and 3 because whole body vascular washout tends to be incomplete and variable following arterial blood collection.

We did, however, repeat brain-only vascular washout studies to compare the regional distribution of CCL2 and CCL5 uptake in the brain following saline or LPS treatment in male CD-1 mice. We measured uptake in 11 brain regions at a single time point (10 minutes) post-chemokine injection, and all measures were corrected for residual albumin brain/serum ratios in the vascular space. Figure 14 shows that uptake of both CCL2 and CCL5 is widespread in all brain regions studied, and that LPS significantly induces increased brain uptake in most regions; for CCL2, there was a trend (<p=0.1) for LPS increasing brain uptake in midbrain and pons-medulla, and significant differences were not observed for striatum or hippocampus, although there were increased means. For CCL5, there was a trend for LPS increasing brain uptake in striatum, thalamus, and hippocampus, and a significant effect was not observed for midbrain, although the means were increased.

**Discussion:**

CCL2 and CCL5 contribute to neuroinflammation following inflammatory or traumatic insults, and in chronic neurodegenerative diseases (Mahad and Ransohoff, 2003; Semple et al., 2010a; Semple et al., 2010b; Kiyota et al., 2013; Pittaluga, 2017; Joy et al., 2019). Both chemokines can be harmful to the brain under pathological conditions (Semple et al., 2010a; Joy et al., 2019), highlighting their potential as therapeutic targets. One mechanism by which CCL2 and CCL5 could contribute to harmful effects on the brain is through their interactions with the BBB. Prior work has shown that CCL2 contributes to BBB disruption (Song and Pachter, 2004; Stamatovic et al., 2005; Dimitrijevic et al., 2006), and both CCL2 and CCL5 can regulate
leukocyte trafficking across the BBB (Dzenko et al., 2005; Ubogu et al., 2006). In vitro studies have also shown that CCL2 and CCL5 can cross the BBB in the brain-to-blood direction (Ge et al., 2008; Minten et al., 2014). However, blood-to-brain transport of CCL2 and CCL5 could also be an important mechanism by which these chemokines accumulate in the brain, especially under conditions of systemic inflammation.

Our present study demonstrated that blood-borne CCL2 and CCL5 can cross the intact mouse BBB. Both chemokines exhibited weak binding to the brain endothelial lumen that was eliminated by vascular washout, but had different uptake patterns. We speculate that CCL2 and CCL5 bind differently to BBB glycocalyx HS. HS is a complex, structurally diverse molecule; the repeating uronic acid (either glucuronic acid or L-iduronic acid) and D-glucosamine disaccharides can be modified by N-acetylation and N- or O-sulfation, and this diversity of the HS chain regulates ligand binding (Esko and Lindahl, 2001). Distinct HS structural domains have been implicated in binding of CCL2 and CCL5 (Sweeney et al., 2006; Singh et al., 2015; Jain et al., 2021), but the composition of BBB glycocalyx HS has not yet been characterized.

Both CCL2 and CCL5 partitioned predominantly in brain parenchyma, showing that circulating CCL2 and CCL5 could contribute to CCL2 and CCL5 pools in the brain. Although there has been much focus on how local production of CCL2 and CCL5 by brain cells such as astrocytes, endothelial cells, and neurons mediate neuroinflammation and brain injury (Flugel et al., 2001; Ge et al., 2009; Howe et al., 2017; Pittaluga, 2017), our results indicate that CCL2 and CCL5 from the circulation may also contribute through BBB transport. This is further supported by findings that peripherally administered neutralizing antibodies against CCL2 and CCL5, which have poor brain penetration, have neuroprotective effects in various models of infection.
and inflammation (Kennedy et al., 1998; Denes et al., 2010). We posit that the neutralizing antibodies in these studies effectively blocked CCL2 and CCL5 interactions with the BBB, including their transport across the BBB. Once in the brain, CCL2 and CCL5 could contribute to aspects of neuroinflammation. Further studies are needed to identify the relative contributions of circulating vs. CNS-produced cytokines on neuroinflammatory responses.

We further found that for both CCL2 and CCL5, brain uptake was not saturable by unlabeled CCL2 or CCL5. The absence of saturable blood-to-brain transport has been observed for another chemokine, CCL11, although brain-to-blood transport was found to be saturable (Erickson et al., 2014). Further, radioactively labeled CCL2 has been used to characterize saturable binding sites at the abluminal (brain-facing) surface of isolated brain microvessels (Ge et al., 2008), suggesting that modifications of radioactive labeling would not affect saturable binding. One explanation for the apparent non-saturability could be a high capacity transport system, since HS is abundant on the brain endothelial cell surface (Reitsma et al., 2007).

We next evaluated the effects of RS504393 and Maraviroc, which are inhibitors of CCL2-CCR2 and CCL5-CCR5 binding, respectively (Mirzadegan et al., 2000; Kondru et al., 2008). Neither inhibitor had a significant effect on chemokine uptake, suggesting that CCR2 and CCR5 do not mediate the brain uptake of CCL2 or CCL5. In contrast, both heparin and eprodisate significantly inhibited the transport of CCL2, and the vascular binding of CCL5, suggesting a predominant role for heparan sulfates in the binding and transport of both chemokines. Heparan sulfates and their respective proteoglycan core proteins are abundant in the endothelial glycocalyx (Reitsma et al., 2007), and can function as endocytic receptors, as has been shown for HSPGs such as syndecans and glypicans (Christianson and Belting, 2014). The brain endothelial
glycocalyx has been shown to be extensive (Ando et al., 2018), suggesting an abundance of HS binding sites at the brain endothelial cell lumen. Little is currently known about the contributions of HS to transcytosis at the BBB, although HS has been shown to regulate the passage of other substances across the BBB, such as gp120/HIV-1 (Banks et al., 1998; Banks et al., 2004), basic fibroblast growth factor and exosomes (Deguchi et al., 2002; Joshi and Zuhorn, 2021). Further, the abluminal binding of CCL2 to brain endothelial cells is HS-independent (Andjelkovic et al., 1999), indicating that distinct mechanisms regulate its blood-to-brain vs. brain-to-blood transport.

Our finding that eprodisate inhibited the brain uptake of CCL2 and CCL5 suggests a novel mechanism for this drug. Eprodisate was initially developed to inhibit the binding of amyloids to glycosaminoglycans (GAGs), and it was found to inhibit development of systemic amyloidosis caused by serum amyloid A in mice (Kisilevsky et al., 1995). Eprodisate has also undergone clinical trials for the treatment of renal amyloidosis (Dember et al., 2007). Our findings in situ suggest that eprodisate can inhibit chemokine-GAG interactions as well as those with GAGs and amyloids. One limitation of this study is that we only assayed the ability of heparin and eprodisate to inhibit the brain uptake of CCL2 and CCL5 in situ, and future work is needed to determine if these inhibitory effects would occur with therapeutic dosing. Another limitation is that heparin and eprodisate do not inform aspects of molecular specificity such as the optimal HS composition for chemokine binding or the identity of the transporter, which is likely a membrane-bound HSPG. However, our results indicate that interactions of CCL2 and CCL5 with HS at the BBB glycocalyx facilitate their brain uptake.
Finally, we showed that BBB transport of CCL2 and CCL5 was markedly increased in most brain regions with systemic inflammation induced by LPS. When compared with peripheral organs, the uptake and vascular binding of both CCL2 and CCL5 was much lower in the brain under basal and LPS-stimulated conditions. However, we found that LPS did not induce global increases in chemokine binding and uptake in peripheral organs, but that the brain, liver, and kidney were selectively affected. Both chemokines regulate systemic and neuroinflammatory responses; although their activities are complex. For example, CCL2 knockout mice produce increased concentrations of IL-1β and TNFα in blood, but lower levels of those cytokines in brain following treatment with a 5mg/kg dose of LPS. Brain concentrations of other pro-inflammatory chemokines and reactive microgliosis were also attenuated following LPS in CCL2 knockout mice, and there was reduced HPA axis activation as measured by blood corticosterone (Thompson et al., 2008). Systemic CCL2 induction protects against sepsis-associated mortality by promoting macrophage-mediated bacterial clearance (Gomes et al., 2013). In the brain, activation of the CCL2/CCR2 axis mediates stroke progression, but CCL2 upregulation is also necessary for protective effects of hypoxic preconditioning against stroke (Stowe et al., 2012). CCL5 has diverse functions in the brain that include neurotrophic functions, metabolic regulation, and promoting learning and memory (Lanfranco et al., 2017; Ajoy et al., 2021), but is also implicated in the pathology of multiple sclerosis (Pittaluga, 2017). Both CCL2 and CCL5 are upregulated by multiple cell types in the brain following inflammatory insults, but our results suggest that CCL2 and CCL5 derived from the blood may also contribute to neuroimmune responses. The relative contributions of CNS vs. blood-derived CCL2 and CCL5 on neuroinflammatory responses have yet to be determined, although recent work has suggested that CCL5 mediates microglial recruitment to the vasculature within 24 hours of a
single IP LPS injection (Haruwaka et al., 2019), consistent with our findings that CCL5 transport is upregulated at this timepoint. Presently, we do not know the mechanism(s) regulating the increased chemokine transport across the BBB with LPS, although brain endothelial cells are highly responsive to immune signals (Erickson and Banks, 2018), and may change expression or function of HSPGs or the composition of glycocalyx HS with inflammation. Future work is warranted to determine the contribution of BBB transport to CCL2, CCL5 and other chemokines with important functions in the CNS.

In conclusion, our work identifies a novel blood-to-brain transport mechanism for CCL2 and CCL5, and suggests that transport of CCL2 and CCL5 across the BBB is one mechanism by which the neuroinflammatory response to a systemic insult is regulated. In addition, our results suggest that inhibiting HS-CCL2/CCL5 interactions at the BBB could prevent the accumulation of blood-derived CCL2 and CCL5 in the brain. Future work is needed to determine whether heparin or HS mimetics like eprodisate can protect against neuroinflammation and associated damage in disease states.

Acknowledgements: We thank Ms. Kim Hansen for her management of the Banks/Erickson labs and the Seattle VA Puget Sound Animal Research Facility Staff for providing outstanding care for our animals.

Authorship contributions:

Participated in research design: Quaranta, Erickson, Logsdon, Banks, Reed

Conducted experiments: Quaranta, Weaver, Baumann, Fujimoto, Williams, Kim, Erickson, Logsdon, Omer
Contributed new reagents or analytic tools: Erickson, Banks

Performed data analysis: Quaranta, Erickson, Weaver, Kim

Wrote or contributed to the writing of the manuscript: Erickson, Quaranta, Banks, Logsdon, Reed, Williams
References


effects on blood brain barrier permeability induced by systemic inflammation. *Nat Commun* **10**:5816.


Footnotes

This work was supported by the Veterans Administration (W.A.B.) and the Joe W. and Dorothy Dorsett Brown Foundation (M.A.E.)

Some of this work has been presented previously as an abstract titled “Transport of the Chemokines CCL5 and CCL2 Across the Mouse Blood-Brain Barrier under Physiological and Inflammatory Conditions” at the Experimental Biology 2021 Meeting

No author has an actual or perceived conflict of interest with the contents of this article

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**Figure legends:**

**Figure 1.** Autoradiography of $^{125}$I-labeled (left) and SYPRO Ruby staining of unlabeled (right) CCL2 and CCL5. Both the labeled and unlabeled chemokines appeared as a single band at the expected molecular weights, as reported by the manufacturer.

**Figure 2.** Clearance of $^{125}$I-CCL2 and $^{99m}$Tc-Alb from blood, and $^{125}$I-CCL2 and $^{99m}$Tc-Alb brain uptake with and without washout. Clearance of $^{125}$I-CCL2 from blood was analyzed by non-linear regression (one-phase decay) (A); open circles are mice that received vascular washout after blood collection and closed circles are mice that did not receive washout. $^{99m}$Tc-Alb clearance by linear regression (B) for calculation of exposure time; open squares are mice that received vascular washout after blood collection and closed squares did not receive washout. Exposure times for both $^{125}$I-CCL2 and $^{99m}$Tc-Alb were calculated using the combined clearance curves from washout and no washout groups. Brain uptake of $^{125}$I-CCL2 is shown in (C), and each point was corrected for luminal vascular space by subtracting the $^{99m}$Tc-Alb brain/serum ratios. Brain uptake of co-injected $^{99m}$Tc-Alb is shown in (D). The corresponding data and statistical analysis for this figure are presented in Table 1.

**Figure 3.** Clearance of $^{125}$I-CCL5 and $^{99m}$Tc-Alb from blood, and $^{125}$I-CCL5 and $^{99m}$Tc-Alb brain uptake with and without washout. Clearance of $^{125}$I-CCL5 from blood was analyzed by non-linear regression (one-phase decay) (A); open circles are mice that received vascular washout after blood collection and closed circles are mice that did not receive washout. $^{99m}$Tc-Alb clearance by linear regression (B) for calculation of exposure time; open squares are mice that received vascular washout after blood collection and closed squares did not receive washout. Exposure times for both $^{125}$I-CCL5 and $^{99m}$Tc-Alb were calculated using the combined clearance curves.
curves from washout and no washout groups. Brain uptake of $^{125}$I-CCL5 is shown in (C), and each point was corrected for luminal vascular space by subtracting the $^{99m}$Tc-Alb brain/serum ratios. Brain uptake of co-injected $^{99m}$Tc-Alb is shown in (D). The corresponding data and statistical analysis for this figure are presented in Table 2.

**Figure 4.** Testing $^{125}$I-CCL2 and $^{125}$I-CCL5 for saturability. Clearance of $^{125}$I-CCL2 and $^{125}$I-CCL5 from blood was analyzed by non-linear regression (one-phase decay) (A and B); open crossed circles are mice that received 1µg of unlabeled CCL2 or CCL5 and closed circles are mice that did not receive unlabeled chemokine. Brain uptake of $^{125}$I-CCL2 and $^{125}$I-CCL5 is shown in (C) and (D), respectively. Each point was corrected for luminal vascular space by subtracting the $^{99m}$Tc-Alb brain/serum ratios. Brain uptake of co-injected $^{99m}$Tc-Alb is shown in (D). The corresponding data and statistical analysis for this figure are presented in Table 3.

**Figure 5.** Capillary and parenchymal partitioning of $^{125}$I-CCL2 (A) and $^{125}$I-CCL5 (B) compared with $^{99m}$Tc-Alb. Mice were co-injected with $^{125}$I-chemokine and $^{99m}$Tc-Alb and tissues were collected after 30 minutes of circulation. Group comparisons were capillary vs. parenchyma in each group, by Sidak’s multiple comparisons test. N=4/group, ***p<0.001.

**Figure 6.** Effects of CCR2 and CCR5 inhibitors on the brain uptake of $^{125}$I-CCL2 and $^{125}$I-CCL5 in situ. Mice were co-perfused with a $^{125}$I-chemokine and $^{99m}$Tc-Alb with 10uM of the corresponding inhibitor (RS504393 for CCL2 and Maraviroc for CCL5, open triangles) or DMSO vehicle (open circles). Each point was corrected for luminal vascular space by subtracting the $^{99m}$Tc-Alb brain/perfusate ratios. The corresponding data and statistical analysis for this figure are presented in Table 4.
Figure 7. Effects of heparin and eprodisate on the brain uptake of $^{125}$I-CCL2 and $^{125}$I-CCL5 in situ. Mice were co-perfused with $^{125}$I-chemokine and $^{99m}$Tc-Alb with 20U/ml of heparin (open diamonds) or 50mM eprodisate (open hexagons) and compared to vehicle (closed circles). The same vehicle group was used for comparison to heparin and eprodisate, but is shown as separate figures for clarity. Each point was corrected for luminal vascular space by subtracting the $^{99m}$Tc-Alb brain/perfusate ratios. The corresponding data and statistical analysis for this figure are presented in Table 5.

Figure 8. Effects of heparin on the brain uptake of $^{125}$I-LEL in situ. Mice were co-perfused with $^{125}$I-LEL and $^{131}$I-Alb with 20U/ml of heparin (open diamonds) and compared to vehicle (closed circles). Each point was corrected for luminal vascular space by subtracting the $^{131}$I-Alb brain/perfusate ratios. The corresponding data and statistical analysis for this figure are presented in Table 7.

Figure 9. Dose-responsiveness of $^{125}$I-CCL2 and $^{125}$I-CCL5 brain uptake in situ to eprodisate. Mice were co-perfused with $^{125}$I-chemokine and $^{99m}$Tc-Alb with 0.5, 5, and 50mM eprodisate. Each point was corrected for luminal vascular space by subtracting the $^{99m}$Tc-Alb brain/perfusate ratios. N=8-9 mice per group, *p<0.05, ***p<0.001 vs. zero concentration.

Figure 10: Effects of 0.3mg/kg LPS on $^{125}$I-CCL2 and $^{125}$I-CCL5 brain uptake in situ. Mice were co-perfused with $^{125}$I-chemokine and $^{99m}$Tc-Alb 28 hours after IP injection of saline or LPS. A) Brain uptake of $^{125}$I-CCL2 in male and female CD-1 mice corrected for $^{99m}$Tc-Alb vascular space, B) vascular space of $^{99m}$Tc-Alb co-injected with $^{125}$I-CCL2, C) Brain uptake of $^{125}$I-CCL5 in female CD-1 mice corrected for $^{99m}$Tc-Alb vascular space, D) vascular space of $^{99m}$Tc-Alb co-injected with $^{125}$I-CCL5. N=5-6 mice per group, *p<0.05, ***p<0.001 vs. saline vehicle.
Figure 11: Effects of 0.3mg/kg LPS on $^{131}$I-albumin vascular space and uptake in the whole brain and peripheral organs in male and female CD-1 mice. Clearance of $^{131}$I-albumin from blood was analyzed by linear regression in male (A) and female (B) mice and exposure time was calculated from these values separately for each sex and treatment. The curves from which $K_i$ (organ uptake) and $V_i$ (vascular space) are calculated are shown for brain (C), lung (D), liver (E), kidney (F) and spleen (G). The circles represent the saline group, and squares represent mice treated with LPS. Closed circles are from male mice, and open circles are from female mice. The corresponding data and statistical analysis for this figure are presented in Table 8.

Figure 12: Effects of 0.3mg/kg LPS on $^{125}$I-CCL2 vascular space and uptake in the whole brain and peripheral organs in male and female CD-1 mice. Clearance of $^{125}$I-CCL2 from blood was analyzed by non-linear regression (one phase decay) in male (A) and female (B) mice and exposure time was calculated from these values separately for each sex and treatment. The curves from which $K_i$ (organ uptake) and $V_i$ (vascular space) are calculated are shown for brain (C), lung (D), liver (E), kidney (F) and spleen (G). The circles represent the saline group, and squares represent mice treated with LPS. Closed circles are from male mice, and open circles are from female mice. The corresponding data and statistical analysis for this figure are presented in Table 9.

Figure 13: Effects of 0.3mg/kg LPS on $^{125}$I-CCL5 vascular space and uptake in the whole brain and peripheral organs in male and female CD-1 mice. Clearance of $^{125}$I-CCL5 from blood was analyzed by non-linear regression (one phase decay) in male (A) and female (B) mice and exposure time was calculated from these values separately for each sex and treatment. The curves from which $K_i$ (organ uptake) and $V_i$ (vascular space) are calculated are shown for brain
(C), lung (D), liver (E), kidney (F) and spleen (G). For lung and liver, only the linear portion of the curve was analyzed, but all data points are shown. The circles represent the saline group, and squares represent mice treated with LPS. Closed circles are from male mice, and open circles are from female mice. The corresponding data and statistical analysis for this figure are presented in Table 9.

Figure 14: Effects of 0.3mg/kg LPS on the regional brain uptake of $^{125}$I-CCL2 and $^{125}$I-CCL2 in male CD-1 mice. Brains and serum were collected after a 10 minute circulation time, and the brains were dissected into 11 regions which include the olfactory bulb (OB), striatum (ST), frontal cortex (FC), hypothalamus (HY), thalamus (TH), hippocampus (HC), parietal cortex (PC), occipital cortex (OC), cerebellum (CB), midbrain (MB), and pons-medulla (PM). The tissue/serum ratios were determined for each region at the single timepoint. Differences in means were determined using Sidak’s multiple comparisons test. *p < 0.05, +p< 0.1 (trend), n= 5-7/group.
### Tables:

#### Table 1

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<th>125I-CCL2 Washout</th>
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<td><strong>Ki (µl/g-min)</strong></td>
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**Table 1.** Brain Uptake of 125I-CCL2 and 99mTc-albumin. Ki = slope and Vi = Y-intercept ± SEM. Significantly non-zero slopes are indicated by ##(p<0.01) and significant differences in the Y-intercepts vs. no washout are indicated by *** (p<0.001).

#### Table 2

<table>
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<td><strong>Ki (µl/g-min)</strong></td>
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<td><strong>Vi (µl/g)</strong></td>
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**Table 2.** Brain Uptake of 125I-CCL5 and 99mTc-albumin. Ki = slope and Vi = Y-intercept ± SEM. Significantly non-zero slopes are indicated by ##(p<0.01) and significant differences in the Y-intercepts vs. no washout are indicated by *** (p<0.001).
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<td><strong>Ki (µl/g-min)</strong></td>
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<td><strong>Vi (µl/g)</strong></td>
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<td>27.07 ± 1.61</td>
</tr>
<tr>
<td><strong>R squared</strong></td>
<td>0.08455</td>
<td>0.1906</td>
<td>0.4565</td>
<td>0.792</td>
</tr>
</tbody>
</table>

**Table 3.** Saturability of $^{125}$I-CCL2 and $^{125}$I-CCL5 brain uptake. $Ki$ = slope and $Vi$ = Y-intercept ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>$^{125}$I-CCL2</th>
<th>$^{125}$I-CCL5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ki (µl/g-min)</strong></td>
<td>4.073 ± 0.86###</td>
<td>2.701 ± 0.7871##</td>
</tr>
<tr>
<td><strong>Vi (µl/g)</strong></td>
<td>12.09 ± 5.011</td>
<td>11.85 ± 4.437</td>
</tr>
<tr>
<td><strong>R squared</strong></td>
<td>0.5164</td>
<td>0.3706</td>
</tr>
</tbody>
</table>

**Table 4.** Effects of CCR2 (RS504393) and CCR5 (Maraviroc) inhibitors on $^{125}$I-CCL2 and $^{125}$I-CCL5 brain uptake in situ. $Ki$ = slope and $Vi$ = Y-intercept ± SEM. Significantly non-zero slopes are indicated by ###(p<0.001).
<p>| | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125I-CCL2</td>
<td></td>
<td></td>
<td>125I-CCL5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>Heparin</td>
<td>Eprodisate</td>
<td>Vehicle</td>
<td>Heparin</td>
<td>Eprodisate</td>
<td>Vehicle</td>
<td>Heparin</td>
<td>Eprodisate</td>
</tr>
<tr>
<td>Ki (µl/g-min)</td>
<td>4.145 ± 0.8037###</td>
<td>0.3328 ± 0.421***</td>
<td>0.4199 ± 0.167#, ***</td>
<td>10.81 ± 3.986###</td>
<td>2.589 ± 0.9622###, ^</td>
<td>1.428 ± 0.4199</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vi (µl/g)</td>
<td>0.4023 ± 5.83 ± 11.85 ±</td>
<td>4.328</td>
<td>2.376</td>
<td>4.437</td>
<td>22.31</td>
<td>5.386***</td>
<td>4.686</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.424</td>
<td>0.04586</td>
<td>0.3649</td>
<td>0.424</td>
<td>0.4199</td>
<td>0.2602</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R squared</td>
<td>0.7074</td>
<td>0.04586</td>
<td>0.3649</td>
<td>0.424</td>
<td>0.4199</td>
<td>0.2602</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5**: Effects of heparin and eprodisate on 125I-CCL2 and 125I-CCL5 brain uptake in situ.

Ki= slope and Vi = Y-intercept ± SEM. Significantly non-zero slopes are indicated by #(p<0.05) and ###(p<0.001). Significant differences from vehicle are indicated by *(p<0.05) and ***(p<0.001). ^ indicates a trend vs. vehicle, p= 0.0586.

<p>| | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125I-CCL2</td>
<td></td>
<td></td>
<td>125I-CCL5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>5 min</td>
<td>10 min</td>
<td>30 min</td>
<td>PC</td>
<td>5 min</td>
<td>10 min</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>93.46 ± 0.8622</td>
<td>96.72 ± 3.276</td>
<td>92.41 ± 4.927</td>
<td>54.65 ± 2.382</td>
<td>91.02 ± 2.519</td>
<td>81.51 ± 2.613</td>
<td>50.11 ± 8.194</td>
<td>35.53 ± 3.167</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>97.93 ± 0.0422</td>
<td>N/A</td>
<td>99.07 ± 0.1819</td>
<td>N/A</td>
<td>85.30 ± 0.6708</td>
<td>N/A</td>
<td>94.60 ± 0.3360</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6**: Acid precipitation of 125I-CCL2 and 125I-CCL5 from serum (in vivo) and brain (in situ). Numbers are percentages of the total 125I detected in the pellet, reflecting the percentage of intact chemokine in tissue ± SEM, n=2-3 per group.
<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ki (µl/g-min)</strong></td>
<td>10.14 ± 1.167###</td>
<td>11.72 ± 2.023#</td>
</tr>
<tr>
<td><strong>Vi (µl/g)</strong></td>
<td>48.14 ± 7.185</td>
<td>38.42 ± 7.750</td>
</tr>
<tr>
<td><strong>R squared</strong></td>
<td>0.8676</td>
<td>0.8935</td>
</tr>
</tbody>
</table>

**Table 7:** Effects of heparin on $^{125}$I-LEL brain uptake in situ. $\text{Ki} =$ slope and $\text{Vi} =$ Y-intercept ± SEM. Significantly non-zero slopes are indicated by #($p<0.05$) and ###($p<0.001$). Significant differences from vehicle are indicated by *(p<0.05) and ***(p<0.001).*
<table>
<thead>
<tr>
<th>Organs</th>
<th>131I-albumin</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>LPS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ki (ul/g-min)</td>
<td>Vi (ul/g)</td>
<td>Ki (ul/g-min)</td>
</tr>
<tr>
<td></td>
<td>(R-squared)</td>
<td>(R-squared)</td>
<td></td>
</tr>
<tr>
<td>Whole Brain</td>
<td>0.04360 ± 0.09008 (0.008600)</td>
<td>8.653 ± 0.5367</td>
<td>-0.05796 ± 0.07642 (0.01945)</td>
</tr>
<tr>
<td>Lung</td>
<td>1.755 ± 1.706 (0.03913)</td>
<td>150.5 ± 10.34</td>
<td>-1.349 ± 1.104 (0.04741)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.5854 ± .5031 (0.04775)</td>
<td>65.93 ± 2.997</td>
<td>0.3882 ± .5336 (0.01734)</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.243 ± 0.6719 (0.3333)**</td>
<td>87.99 ± 4.072</td>
<td>1.304 ± .7552 (0.09039)</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.760 ± 0.6735 (0.3835)##</td>
<td>64.71 ± 4.013</td>
<td>2.797 ± 0.5020 (0.4111)###</td>
</tr>
</tbody>
</table>

**Table 8:** Effects of LPS on the vascular binding and uptake of 131I-albumin into whole brain and peripheral organs. $\text{Ki} =$ slope and $\text{Vi} = \text{Y-intercept}$ ± SEM. Significantly non-zero slopes are indicated by $\#(p<0.05)$, $\##(p<0.01)$, $\###(p<0.001)$. Significant differences from vehicle are indicated by *(p<0.05), ***(p<0.01).
<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ki (ul/g-min)</td>
<td>Ki (ul/g-min)</td>
</tr>
<tr>
<td></td>
<td>(R-squared)</td>
<td>(R-squared)</td>
</tr>
<tr>
<td>Whole Brain</td>
<td>0.1222 ± 1.158</td>
<td>-0.4038 ± 2.288</td>
</tr>
<tr>
<td></td>
<td>(0.000696)</td>
<td>(0.001829)</td>
</tr>
<tr>
<td>Lung</td>
<td>1939 ± 621 (0.394)##</td>
<td>1726 ± 483.2 (0.4148)##</td>
</tr>
<tr>
<td></td>
<td>9044 ± 5397</td>
<td>9320 ± 4392</td>
</tr>
<tr>
<td>Liver</td>
<td>111.6 ± 50.51 (0.2339)#</td>
<td>225.4 ± 59.3 (0.4594)##</td>
</tr>
<tr>
<td></td>
<td>1812 ± 443.1</td>
<td>1880 ± 545*</td>
</tr>
<tr>
<td>Kidney</td>
<td>89.79 ± 28.87 (0.3767)##</td>
<td>75.4 ± 26.83 (0.3049)#</td>
</tr>
<tr>
<td></td>
<td>619.1 ± 253.3</td>
<td>633.7 ± 243.9</td>
</tr>
<tr>
<td>Spleen</td>
<td>353.1 ± 127.8 (0.3228)#</td>
<td>284.1 ± 67.55 (0.5098)###</td>
</tr>
<tr>
<td></td>
<td>933.5 ± 1121</td>
<td>239.5 ± 620.8</td>
</tr>
</tbody>
</table>

**Table 9:** Effects of LPS on the vascular binding and uptake of $^{125}$I-CCL2 into whole brain and peripheral organs. Ki = slope and Vi = Y-intercept ± SEM. Significantly non-zero slopes are indicated by # (p<0.05) and ## (p<0.001). Significant differences from vehicle are indicated by *(p<0.05) and *** (p<0.001).
<table>
<thead>
<tr>
<th></th>
<th><strong>125I-CCL5</strong></th>
<th></th>
<th><strong>LPS</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ki (ul/g-min)</strong></td>
<td><strong>Vi (ul/g)</strong></td>
<td><strong>Ki (ul/g-min)</strong></td>
<td><strong>Vi (ul/g)</strong></td>
</tr>
<tr>
<td><strong>(R-squared)</strong></td>
<td><strong>(R-squared)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Whole Brain</strong></td>
<td>0.8966 ± 0.4181</td>
<td>32.38 ± 5.662</td>
<td>1.571 ± 1.143</td>
</tr>
<tr>
<td></td>
<td>(0.3382)</td>
<td></td>
<td>(0.1736)</td>
</tr>
<tr>
<td><strong>Lung</strong></td>
<td>2353 ± 853.4&quot;</td>
<td>4416 ± 5399</td>
<td>4057 ± 634.6&quot;&quot;&quot;&quot;</td>
</tr>
<tr>
<td></td>
<td>(0.6032)</td>
<td></td>
<td>(0.8720)</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>359.0 ± 93.7&quot;</td>
<td>1006 ± 592.8</td>
<td>629.6 ± 69.98&quot;&quot;&quot;&quot;&quot;</td>
</tr>
<tr>
<td></td>
<td>(0.7460)</td>
<td></td>
<td>(0.9310)</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td>114.3 ± 27.37&quot;&quot;&quot;&quot;</td>
<td>649.7 ± 370.6</td>
<td>159.9 ± 21.97&quot;&quot;&quot;&quot;&quot;</td>
</tr>
<tr>
<td></td>
<td>(0.6597)</td>
<td></td>
<td>(0.8413)</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>257.8 ± 61.09&quot;&quot;&quot;&quot;</td>
<td>1067 ± 827.3</td>
<td>288.5 ± 23.35&quot;&quot;&quot;&quot;&quot;</td>
</tr>
<tr>
<td></td>
<td>(0.6643)</td>
<td></td>
<td>(0.9385)</td>
</tr>
</tbody>
</table>

**Table 10:** Effects of LPS on the vascular binding and uptake of 125I-CCL5 into whole brain and peripheral organs. **Ki**= slope and **Vi** = Y-intercept ± SEM. Significantly non-zero slopes are indicated by "(p<0.05) and """"(p<0.01). Significant differences from vehicle are indicated by *(p<0.05), **(p<0.01) and ***(p<0.001).
Figure 1

 Autoradiography

 SYPRO Ruby

 CCL2  CCL5

 CCL2  CCL5
Figure 2

A. $^{125}$I-CCL2 Clearance

B. $^{99m}$Tc-Albumin Clearance

C. $^{125}$I-CCL2 Brain Uptake

D. $^{99m}$Tc-Albumin Brain Uptake
Figure 3
Figure 4
**Figure 5**

**A** $^{125}$I-CCL2 Brain Partitioning

<table>
<thead>
<tr>
<th></th>
<th>Albumin</th>
<th>CCL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain/Serum Ratio (μL/g)</td>
<td><img src="chart1.png" alt="Bar chart" /></td>
<td><img src="chart2.png" alt="Bar chart" /></td>
</tr>
</tbody>
</table>

- **Capillary**
- **Parenchyma**

**B** $^{125}$I-CCL5 Brain Partitioning

<table>
<thead>
<tr>
<th></th>
<th>Albumin</th>
<th>CCL5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain/Serum Ratio (μL/g)</td>
<td><img src="chart3.png" alt="Bar chart" /></td>
<td><img src="chart4.png" alt="Bar chart" /></td>
</tr>
</tbody>
</table>

- **Capillary**
- **Parenchyma**

Significance levels indicated by ***.
Figure 6
Figure 7

A. $^{125}$I-CCL2 Brain Uptake

B. $^{125}$I-CCL5 Brain Uptake

C. $^{125}$I-CCL2 Brain Uptake

D. $^{125}$I-CCL5 Brain Uptake

Perfusion Time (min)

Delta Brain/Perfusate Ratio ($\mu$l/g)

Vehicle vs. Heparin (20U/ml)

Vehicle vs. Eprodisate (50mM)
Figure 8
Figure 9

A

$^{125}$I-CCL2 Brain Uptake

Delta Brain/Perfusate Ratio (μL/g)

[Episodesate] (mM)

0  0.5  5.0  50.0

B

$^{125}$I-CCL5 Brain Uptake

Delta Brain/Perfusate Ratio (μL/g)

[Episodesate] (mM)

0  0.5  5.0  50.0

*** *
Figure 10

(A) 

$^{125}$I-CCL2

Delta Brain/Perfusate Ratio (µL/g)

** Male  **  Female

Vehicle  

0.3mg/kg LPS

***

(B) 

$^{131}$I-Albumin

Delta Brain/Perfusate Ratio (µL/g)

** Male  **  Female

Vehicle  

0.3mg/kg LPS

* p=0.0539

(C) 

$^{125}$I-CCL5

Delta Brain/Perfusate Ratio (µL/g)

Vehicle  

0.3 mg/kg LPS

***

(D) 

$^{131}$I-Albumin

Delta Brain/Perfusate Ratio (µL/g)

Vehicle  

0.3 mg/kg LPS

p=0.2132
Figure 11

A) Male $^{131}$I-albumin clearance

B) Female $^{131}$I-albumin clearance

C) $^{131}$I-albumin Brain

D) $^{131}$I-albumin Lung

E) $^{131}$I-albumin Liver

F) $^{131}$I-albumin Kidney

G) $^{131}$I-albumin Spleen

- Saline (M)
- LPS 0.3mg/kg (M)
- Saline (F)
- LPS 0.3mg/kg (F)
Figure 12
Figure 13
Figure 14

A

$^{125}$I-CCL2 Brain Regions

Delta Tissue/ Serum Ratio (µl/g)

- Saline
- LPS 0.3mg/kg

OB, ST, FC, HY, TH, HC, PC, OC, CB, MB, PM

WB LPS

WB Sal

B

$^{125}$I-CCL5 Brain Regions

Delta Tissue/ Serum Ratio (µl/g)

- Saline
- LPS 0.3mg/kg

OB, ST, FC, HY, TH, HC, PC, OC, CB, MB, PM

WB LPS

WB Sal