EFFECT OF PERIPHERAL INFLAMMATORY PAIN ON THE BLOOD-BRAIN BARRIER

by

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"A teacher affects eternity; he can never tell where his influence stops."
Henry Adams
The Education of Henry Adams, 1907

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DEDICATION

This work is dedicated to my parents, Van and Kim-Tuy Hau.

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TABLE OF CONTENTS

TABLE OF CONTENTS - *CONTINUED*

TABLE OF CONTENTS - *CONTINUED*

TABLE OF CONTENTS – *CONTINUED*

LIST OF FIGURES

CHAPTER 1. GENERAL INTRODUCTION

CHAPTER 2. EFFECT OF INFLAMMATORY PAIN ON BLOOD-BRAIN BARRIER DRUG DELIVERY

CHAPTER 3. EFFECT OF INFLAMMATORY PAIN ON ANALGESIA

LIST OF FIGURES - *CONTINUED*

LIST OF TABLES

CHAPTER 2. EFFECT OF INFLAMMATORY PAIN ON BLOOD-BRAIN BARRIER DRUG DELIVERY

CHAPTER 4. EFFECT OF INFLAMMATORY AGENTS DIRECTLY ON THE BLOOD-BRAIN BARRIER

CHAPTER 5. EFFECT OF INFLAMMATORY PAIN ON BLOOD-BRAIN BARRIER TIGHT JUNCTION PROTEIN EXPRESSION

ABSTRACT

Currently, there is a growing body of research characterizing the blood-brain barrier (BBB) under normal physiological conditions; however, little is known about BBB regulation under pathophysiological conditions, such as inflammatory pain. This dissertation elucidates peripheral inflammatory pain effects on the BBB both functionally in terms of permeability and structurally via tight junction (TJ) protein expression and regulation.

Inflammation was produced by subcutaneous injection of formalin, λ-carrageenan, or complete Freund's adjuvant (CFA) into the right hind paw of rats. *In situ* perfusion and Western blot analyses were performed to assess BBB integrity after inflammatory insult. *In situ* brain perfusion determined that peripheral inflammation significantly increased the uptake of a membrane impermeant marker, sucrose into the cerebral hemispheres in all inflammatory models. Subsequently, a 0-168h time course study of λ-carrageenan-induced inflammatory pain elicited a biphasic increase in BBB permeability of sucrose with the first phase occurring from 1-6h and the second phase occuring at 48h. λ-carrageenan-induced inflammatory pain also increased brain uptake of a commonly used analgesic, codeine at the same time-points. This is the first known observation that peripheral inflammation results in greater analgesic drug uptake to the brain. This uptake also correlated with its antinociceptive profile over a 168h time course. This suggests the presence of inflammatory pain may be an important consideration in therapeutic drug dosing, potential adverse effects and/or neurotoxicity.

Western blot analyses showed altered TJ protein expression during peripheral inflammation. Occludin significantly decreased in the λ-carrageenan- and CFA-treated groups. ZO-1 expression was significantly increased in all pain models. Claudin-1 protein expression was present at the BBB and remained unchanged during inflammation. Actin expression was significantly increased in the λ-carrageenan- and CFA-treated groups. Over a 72h time period with λ-carrageenan-induced inflammatory pain, altered TJ protein expression of occludin and ZO-1 correlated with permeability changes in BBB function. This is the first report of peripheral inflammation inducing alterations in TJs and increasing permeability of the BBB. This dissertation demonstrates that changes in the structure of TJs leading to alterations in the BBB may have important clinical ramifications concerning central nervous system homeostasis and therapeutic drug delivery.

CHAPTER 1. INTRODUCTION

1. Morphology and Biochemistry of the Blood-Brain Barrier

1.1 The History of the Blood-Brain Barrier

Optimal brain neuronal function is dependent upon maintenance of brain homeostasis. Slight perturbations of homeostasis can cause profound alterations in mental and neuromotor functioning including mental blunting, seizures and death (Muller and Ackenheil, 1995; Viswambharan et al., 2003; Pavlovsky et al., 2005). Thus, the composition of brain extracellular fluid must be maintained within a precise physiological range, independent of fluctuations within the blood. This maintenance is served by the presence of a blood-brain barrier (BBB).

The BBB is a specialized selective partition between the central nervous system (CNS) and peripheral circulation. It is made of cerebral microvascular endothelial cells with specific transporters and few fenestrations to control passage of substances through endothelial cells. The first time this partition was coined the "blood-brain barrier" was by Lewandowsky, while investigating potassium ferrocyanide penetration into the brain (Lewandowsky, 1900). However, observations of a partition between the CNS and peripheral circulation were first described by Paul Ehrlich and Edwin Goldman in the late 19^{th} and early 20^{th} centuries when they saw water soluble dyes injected into the peripheral circulation did not stain the brain or color the cerebrospinal fluid (CSF) but did color

peripheral tissues. When the dyes were injected centrally into the subarachnoid space, the brain and CSF were colored but not the peripheral tissues (Ehrlich, 1885 1906; Goldman, 1909; Goldman, 1913). Friedemann in 1942 then showed highly lipid soluble basic dyes injected peripherally stained the brain and thus were able to cross the BBB, indicating charge and lipophilicity are factors in the BBB permeability of a substance (Friedemann, 1942).

In 1941, Broman observed the presence of two brain barrier systems: the BBB at the cerebral microvasculature and the blood-CSF barrier at the choroid plexus. In addition, he suggested the capillary endothelial cells of the BBB were what established the barrier function (Broman, 1941). Later, Reese and Karnovsky in 1967 were able to visualize the BBB with horseradish peroxidase, substantiating that cerebral capillary endothelial cells restricted movement of substances between the blood and brain extracellular fluid through the presence of tight junctions between the cells. They showed that horseradish peroxidase diffused past the astrocytic end feet and basement membrane of the BBB but stopped at the tight junctions of the cerebral endothelial cells (Reese and Karnovsky, 1967).

In 1981, the BBB capillary endothelium was shown to be unique in its tight junctions and barrier properties in comparison to the peripheral capillary network of other organs by Stewart and Wiley (Stewart and Wiley, 1981). They showed that when embryonic quail brain was vascularized by embryonic chick gut vessels, the transplanted vessels expressed BBB physiological characteristics and excluded dyes such as trypan blue. However, when embryonic chick gut was vascularized by embryonic quail brain

vessels, they were leaky to trypan blue and did not maintain BBB characteristics as they would have if they were allowed to develop within the brain. These experiments support the thought that the unique physiological characteristics of the BBB are due to the expression of a distinctive set of genes within the capillary endothelium possibly influenced by cofactors within the surrounding tissue.

1.2 Anatomy and Physiology of the Blood-Brain Barrier Periendothelial Accessory Structures

Figure 1.1 is a three-dimensional representative cross-section of a BBB cerebral capillary. Shown are the periendothelial accessory structures of the BBB including astrocytes and a basal membrane (not shown are pericytes, which would be located between the basal membrane and endothelial cells). The endothelial cells form the lumen of the capillary by encircling the inside of the basal membrane. They are distributed along the length of the vessel. The basal membrane surrounds the pericytes and endothelial cells forming a region between which is known as the Virchow-Robin space. Astrocytes in turn surround the basal membrane with their end foot processes apposing the membrane.

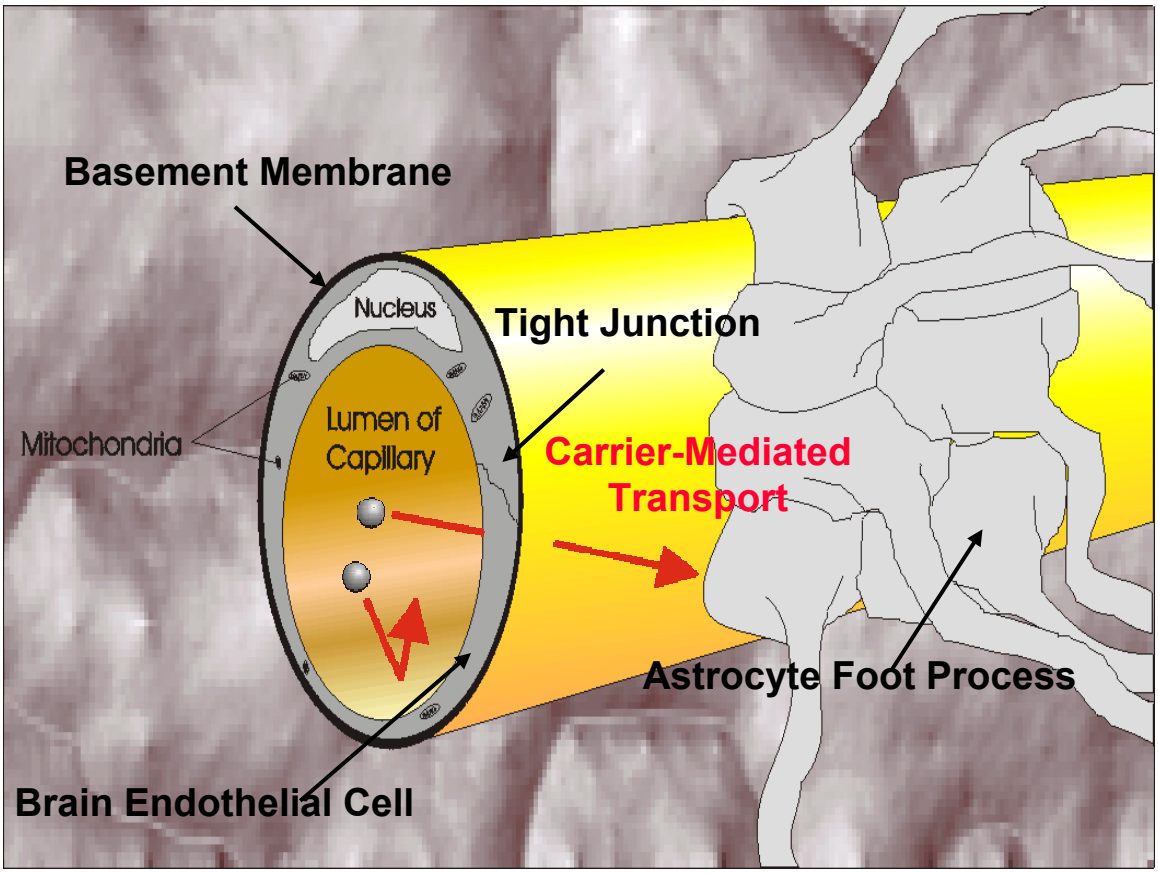

Figure 1.1 A representative cross / longitudinal-section of a cerebral capillary of the BBB. Spheres within the lumen of the capillary represent molecules that in most cases are impermeable to the BBB, however certain nutrients and drugs are able to utilize carrier-mediated transport to cross the BBB.

1.2.1 Pericytes

Peripheral pericytes surrounding capillary walls generally serve as contractile connective tissue cells, however central microvascular pericytes lack an α-actin isoform, typical of contractile cells suggesting BBB pericytes are not involved in capillary contraction (Nehls and Drenckhahn, 1991). Rather, pericytes have been suggested to be involved in regulation of endothelial cell proliferation, survival, migration, differentiation, and vascular branching (Hellstrom et al., 2001). In *in vitro* systems /

models pericytes have been shown to have gap junction communication with endothelial cells (Larson et al., 1997). They are thought to be involved in endothelial cell proliferation, via selective inhibition of endothelial cell growth (Antonelli-Orlidge et al., 1989). In studies where there have been a lack of pericytes, endothelial hyperplasia and abnormal brain vascular morphogenesis have been demonstrated (Hellstrom et al., 2001). Pericytes have also demonstrated the ability to phagocytize exogenous protein from the CNS, suggesting BBB pericytes may be derived from microglia (Coomber and Stewart, 1985). It is also thought that pericytes may play a role in BBB tight junction formation, similar to astrocytes (Minakawa et al., 1991).

1.2.2 Astrocytes

Astrocytes are thought to play a role in determination of BBB morphology, function, and protein expression (Cancilla and DeBault, 1983; Beck et al., 1984; Arthur et al., 1987). They are glial cells that envelop > 99% of the BBB capillary endothelium, serving as scaffolds, guiding neuron and BBB vessel placement during development. Astrocytes act as a bridge associating the cerebral microvasculature with neurons. They have been found to increase BBB marker expression of γ-GTP (Maxwell et al., 1987), cAMP (Rubin et al., 1991), Na+/K+-ATPase (Beck et al., 1986), multidrug-resistance receptors (MDR) (Sobue et al., 1999), and GLUT-1 transporters (Pardridge et al., 1990; Boado et al., 1994). In addition, bovine brain microvascular endothelial cell (BBMEC) monolayers when co-cultured with astrocytes or cultured in astrocyte-conditioned media express greater TJ complexity and BBB markers (Arthur et al., 1987; Tao-Cheng et al.,

1987). Astrocyte influence include actin filament migration and redistribution at TJs (Gaillard et al., 2001), increased glucose transport (Maxwell et al., 1989), increased transendothelial resistance (a measure of paracellular permeability) (Dehouck et al., 1990; Rubin et al., 1991), and decreased fluid-phase endocytosis. In addition, because of the sheath the astrocytes form around the capillary, it is sometimes thought astrocytes may contribute to the physical barrier of the BBB, however, this is unlikely as Brightman and Reese in 1969 showed horseradish peroxidase readily diffusing between adjacent astroctyes (Brightman and Reese, 1969).

1.2.3 Basal Membrane

The basal membrane acts as an additional barrier to macromolecule diffusion, provides a foundation for cell attachment, acts as a substratum for cell migration, and separates adjacent tissues. The basal membrane consists of laminin, fibronectin, tenascin, collagens, and proteoglycan (Timpl and Brown, 1996). In addition, integrins, transmembrane receptors, adhere cells to the basal membrane by bridging the cytoskeletal elements of a cell to the extracellular matrix of the membrane (Hynes, 1992).

1.2.4 Neurons

Neurons may also play a role BBB formation. Various *in vivo* and *in vitro* studies have suggested a role of neurons and / or astrocytes in induction of the BBB cerebral microvasculature (Wolburg, 1995; Bauer and Bauer, 2000). Experiments involving coculture of cerebral capillary endothelial cells with neurons versus similar endothelial

cells with glial cells have shown dose-dependent increases in γ-glutamyl transpeptidase activity (a marker for BBB endothelial cells), indicating neuronal inductive effect (Tontsch and Bauer, 1991).

1.3 Anatomy and Physiology of Cerebral Capillary Endothelial Cells

The CNS microvasculature is an extensive network that provides blood flow and nutrients to the brain. It has a surface area of ~ 100 $cm^2 \times g^{-1}$ tissue with capillary and endothelial cell volume comprising of nearly 1% and 0.1% of tissue volume, respectively (Pardridge et al., 1990). The mean intercapillary distance is ~ 40 μm (Duvernoy et al., 1983). Thus, a near instantaneous solute equilibrium throughout the brain interstitial space could be readily achieved by small molecules if it was not for the BBB.

The BBB is differentiated from the peripheral microvasculature in that it possesses many unique characteristics mainly imparted by its endothelial cells. Unique BBB endothelial cell characteristics include tight junctions, decreased pinocytotic activity, increased presence of mitochondria, enzymatic activity, decreased wall thickness, and polarity.

1.3.1 Tight Junctions

BBB microvessel endothelial cells are characterized by their tight junctions (TJ), which form a rate-limiting barrier to paracellular diffusion of substances, keeping the microenvironments of the systemic circulation and the brain distinct. These TJs lead to

high endothelial electrical resistance values in the range of 1500-2000 Ω x cm^2 as compared to 3 – 33 Ω x cm^2 in other peripheral tissues (Crone and Christensen, 1981; Butt et al., 1990) indicating lower paracellular permeability. In addition, expression of TJs correlate with a reduction in aqueous-based paracellular diffusion which is commonly found in other organs (Brightman and Tao-Cheng, 1993).

TJs form a continuous network of parallel, interconnected, intramembrane fibrils that are located at the apexes of endothelial cells (Staehelin, 1973; Schneeberger and Karnovsky, 1976). An increased organization of cytoplasmic fibrils is associated with tissues that have greater electrical resistances and decreased permeability (Claude and Goodenough, 1973; Claude, 1978). These BBB TJs are composed of transmembrane and cytoplasmic proteins linked to an actin-based cytoskeleton forming an impermeant seal while remaining capable of rapid regulation and modulation (Figure 1.2).

There are three main TJ transmembrane integral proteins: claudin, occludin and junction adhesion molecule (JAM) that bind homotypically to their respective protein on an adjacent endothelial cell. The former two are considered the backbone of TJ strands (claudin forms the TJ primary seal while occludin is a TJ regulatory protein) and JAM helps in regulating leukocyte migration (Hirase et al., 1997; Furuse et al., 1999).

In addition, several cytoplasmic accessory proteins including the zonula occludens (ZO-1, -2, and -3) play an important role in maintenance of the BBB. They provide a support structure for signal transduction proteins and act as recognition proteins for TJ placement, binding integral proteins and actin (Haskins et al., 1998).

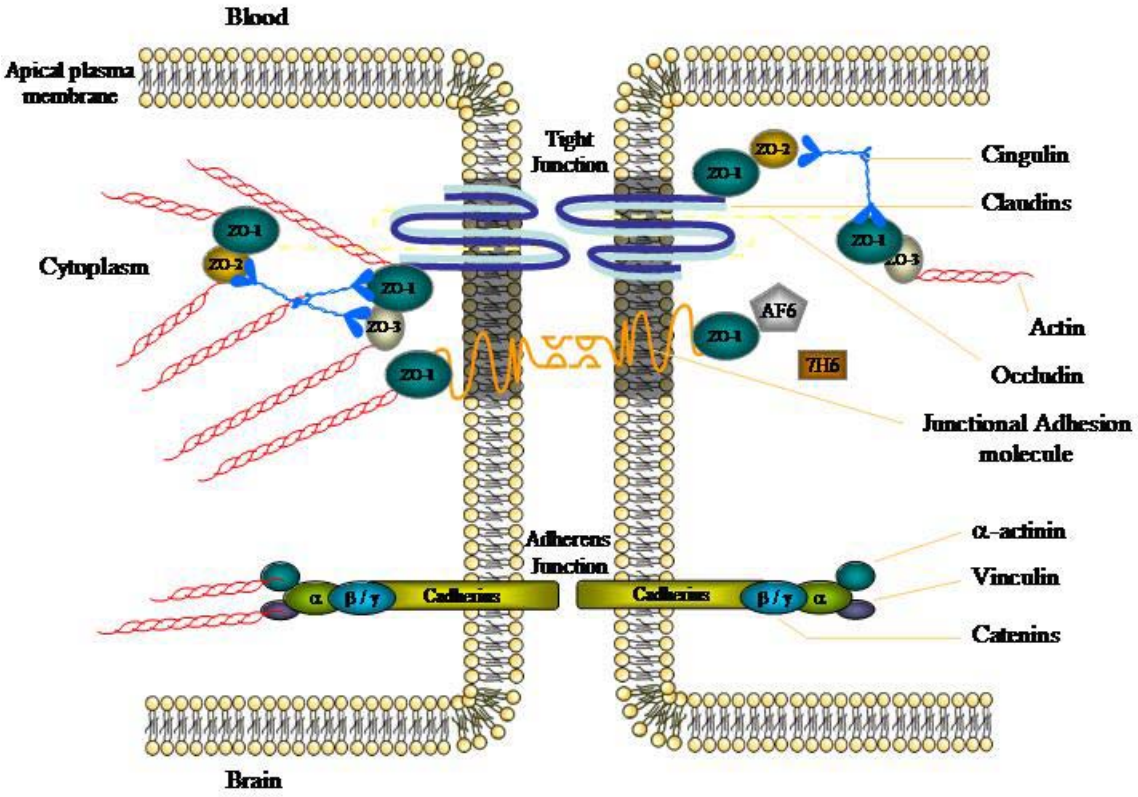

Figure 1.2 Proposed interactions of the major proteins associated with tight junctions (TJs) at the blood–brain barrier (BBB). The TJ is embedded in a cholesterol-enriched region of the plasma membrane (shaded). Claudins make up the backbone of TJ strands by forming dimers and binding homotypically to claudins on adjacent cells to produce the primary seal of the TJ. Claudins comprise a multigene family with 24 isoforms currently identified. Recent studies have identified the presence of claudin 1 and claudin 5 at the BBB (Huber et al., 2001). Occludin functions as a dynamic regulatory protein, whose presence in the membrane is correlated with increased electrical resistance across the membrane and decreased paracellular permeability. The TJ also consists of several accessory proteins necessary to form structural support for the tight junction. The zonula occludens proteins (ZO1, ZO2 and ZO3) belong to a family of proteins known as MAGUKs (membrane-associated guanylate kinase-like proteins). These proteins serve as recognition proteins for tight junctional placement and as a support structure for signal transduction proteins. The known binding patterns of the ZO proteins to one another are shown. AF6 is a Ras effector molecule associated with ZO1. 7H6 antigen is a phosphoprotein found at tight junctions impermeable to ions and macromolecules. Cingulin is a double-stranded myosin-like protein that binds preferentially to ZO proteins at the globular head and to other cingulin molecules at the globular tail. The primary cytoskeletal protein, actin, has known binding sites on all of the ZO proteins, and on claudin and occludin (Huber et al., 2001).

1.3.1.1 Transmembrane Protein: Occludin

Occludin was first identified in avian junctional liver complexes and then later in mammalian species (Furuse et al., 1993; Ando-Akatsuka et al., 1996) It is a 65 kDa integral membrane-bound protein that is composed of four transmembrane domains, two extracellular loops, and a large carboxy-terminal cystolic domain (Furuse et al., 1993). The extracellular loops are distinct from one another with the first loop containing ~60% tyrosine and glycine residues, which is conserved in all animal species, although the function is still unknown. The ~150 amino acid carboxy terminal is also conserved among animal species (Ando-Akatsuka et al., 1996) and is known to bind actin cytoskeleton (Wittchen et al., 1999) and the zonula occludens (cytoplasmic-plaque proteins): ZO-1, -2, and -3 (Furuse et al., 1994; Haskins et al., 1998; Itoh et al., 1999).

Occludin presence has been associated with increased electrical resistance and decreased paracellular permeability (Balda et al., 1996; Huber et al., 2001). Using immunofreeze fracture microscopy, occludin has been found to be concentrated within tight junctional fibrils (Furuse et al., 1993) with a detergent-extractable pool found along the basolateral surface that is not embedded in the membrane (Sakakibara et al., 1997). This intracellular pool of occludin may serve as a reservoir for the dynamic regulation of tight junctional complexity, thereby affecting paracellular permeability (Cordenonsi et al., 1997; Sakakibara et al., 1997). In experiments with cultured epithelial cells, occludin was shown to play a role in regulation of size-selective paracellular diffusion of hydrophilic molecules (Balda et al., 2000).

Several studies have shown occludin playing a functional role in forming the tight junctional barrier. When occludin-null fibroblasts were transfected with occludin, a Ca^{2+}-dependent adhesion was formed between fibroblasts (Furuse et al., 1998). In MDCK cells, an overexpression of occludin resulted in an increased number of TJ strands as well as an increase in transepithelial resistance (Huber et al., 2001). In addition, when the carboxy-terminals of occludin were truncated within MDCK or Xenopus embryo cells, this resulted in a paracellular leakage of low molecular weight tracers (Medina et al., 2000). In studies, with astrocyte-conditioned media (see section *Physiology of the BBB periendothelial accessory structures: astrocytes*) there was found a direct correlation between the expression of occludin and transendothelial resistance of primary cultured porcine brain microvessels (Garcia et al., 2004). However, Saitou et al. observed that occludin knock out mice still displayed well developed TJs (Saitou et al., 2000), indicating while occludin is important for the regulation of the TJ barrier, it is not the primary TJ protein.

1.3.1.2 Transmembrane Protein: Claudin

Because of the paradoxical results obtained from occludin knock out mice, Tsukita et al. searched for other TJ integral components. They eventually discovered claudins, which since their discovery in 1998, the family has expanded to over 24 members (some reported previously under a different name but now recognized as belonging to the claudin family) (Furuse et al., 1998; Tsukita et al., 2001). Claudins are considered to be the primary sealing protein of the TJ. They encode 20-27 kDa proteins

with four transmembrane domains and two extracellular loops similar to occludin but have no structural relation to occludin. Claudins also have a carboxy intracellular tail but is much shorter in comparison to occludin. The amino acids of the tail are highly conserved within the claudin family and all contain PDZ binding motifs (Tsukita et al., 2001). Through the PDZ binding motifs, the claudins are linked to zonula occluden proteins (ZO-1, ZO-2, ZO-3) (Itoh et al., 1999; Gonzalez-Mariscal et al., 2003).

When claudin-1 or claudin-2 were transfected into mouse L-fibroblasts that lacked TJs, a network of TJ strands formed between the cells that was indistinguishable from *in situ* TJ strands (Furuse et al., 1998), thus suggesting claudins formed the backbone of TJ strands. As discussed previously, when occludins were introduced similarly, such well-organized strands were not apparent, however when it was introduced with claudin expressing L transfectants, it was incorporated into the claudin-based strands (Furuse et al., 1998).

Claudin subtypes are varied in their tissue distribution and even within the same organ they may have differing subcellular localization. They have been shown to form heteropolymers as well as bind between adjacent strands in pairs as subtypes both in a homotypic or heterotypic manner (Furuse et al., 1999; Tsukita and Furuse, 2000), thus potentially resulting in numerous permutations of combinations within the same tissue. Of the claudin subtypes, the most interest to this dissertation are claudin-1, -5, and -12. Each of these has been found to be expressed in brain endothelial cells (Morita et al., 1999; Liebner et al., 2000; Nitta et al., 2003). However, a recent unpublished study by Nitta et al. (but discussed in the JCB vol. 161, No.3, May 2003 article, “Size-selective

loosening of the blood-brain barrier in claudin-5-deficient mice" by Nitta et al.) showed anti-claudin-1 antibody staining claudin-1-deficient mice. This may suggest previous studies using the same anti-claudin-1 antibody in brain endothelial cells may be the result of cross-reactivity with claudin-5 or -12. (This study came out after the completion of the dissertation, which is why claudin-1 was studied in chapter IV.) Claudin-5 was recently shown to be a critical determinant of BBB permeability in mice, but not essential to the presence of TJs. Knock out of claudin-5 in mice demonstrated a selective increase in paracellular permeability of small molecules but not a general breakdown of TJs (Nitta et al., 2003). The maintenance of TJ integrity can be attributed to the presence of claudin-12 or the up-regulation of another yet unidentified claudin in mouse brain endothelial cells.

1.3.1.3 Transmembrane Protein: Junctional Adhesion Molecule

JAM (~40 kDa) is another transmembrane protein localized to the endothelial cell TJ (Martin-Padura et al., 1998). It is a member of the immunoglobulin superfamily (IgSF) and has a single transmembrane domain with two extracellular domains with intrachain disulfide bonds. Homotypic binding of JAM between adjacent cells results in a decrease in permeability and a promotion of cell-cell adhesion (Martin-Padura et al., 1998). Like occludin, studies of exogenously expressed JAM in L transfectants do not result in a reconstitution of TJs but do associate laterally with the claudin-based backbone of TJ strands. JAM has also been shown in Chinese hamster ovary transfectants to facilitate the junctional localization of ZO-1 and occludin and in addition, can be co-

precipitated with cingulin and ZO-1 in the insoluble fraction of Caco-2 cells (Bazzoni et al., 2000). Like claudin, JAM also has PDZ-binding motifs on the carboxy terminal that allow it to bind to ZO-1 (Bazzoni et al., 2000). While the exact function of JAM is still uncertain, it is thought that it plays a role in controlling the movement of leukocytes through TJs as well as in the molecular architecture of the TJ through its interaction with ZO-1, cingulin and occludin (Martin-Padura et al., 1998; Bazzoni et al., 2000).

1.3.1.4 Cytoplasmic Protein: Zonula Occluden

In addition to the transmembrane proteins, there are several accessory proteins that are important in the formation, maintenance, and regulation of tight junctions: zonula occludens (ZO-1, ZO-2, and ZO-3), cingulin, AF6, and 7H6. ZO proteins are members of a family of membrane-associated guanylate kinase-like homologues, which are important in site recognition, signal transduction, and structural support (Jesaitis and Goodenough, 1994; Haskins et al., 1998; Tsukamoto and Nigam, 1999). They contain multiple PDZ domains, a src homology 3 domain, and guanylate kinase-like (GUK) domain. This allows them to bind carboxy-terminal cytoplasmic ends of transmembrane proteins, signaling proteins, and cytoskeletal factors.

Of the ZO proteins, ZO-1 (220 kDa) is the most characterized of these accessory proteins and has binding sites for occludin, claudin, JAM, ZO-2, ZO-3, cingulin, and actin, which allows it to maintain and regulate tight junctional structure (Stevenson et al., 1986; Fanning et al., 1998). ZO-1 is localized at TJs in both epithelial and endothelial cells in addition to adherens junctions in non-TJ containing cells such as fibroblasts and

intercalacted disks of cardiac myocytes (Wolburg and Lippoldt, 2002). The carboxy-terminal portion of ZO-1 also directly binds cytoskeleton actin filaments, possibly accounting for the cross-linking of occludin to actin at TJs, supporting the importance of ZO-1 in regulating TJs (Fanning et al., 1998).

ZO-2 (160 kDa) has been localized to both TJs and adherens junctions (Jesaitis and Goodenough, 1994; Itoh et al., 1999), thus suggesting a role in TJ maintenance and regulation as well. In addition, the second PDZ domain of ZO-2 has been shown to bind ZO-1, forming a heterodimer (Itoh et al., 1999).

ZO-3 (130 kDa) also localized to TJs in *in vitro* binding studies has demonstrated binding to occludin, claudin and ZO-1 but not ZO-2 (Haskins et al., 1998; Itoh et al., 1999).

1.3.1.5 Cytoplasmic Protein: Cingulin

Cingulin (140 kDa) is a heat-stable elongated dimer composed of two polypeptides (Citi et al., 1988; Cordenonsi et al., 1999) localized to the endofacial surface of the TJ complex (Citi et al., 1988). It is similar in structure to myosin with a globular head and tail domains in addition to an α-helical rod (Cordenonsi et al., 1999). *In vitro* studies showed ZO-2, ZO-3, F-actin, myosin, JAM, and AF6 interacts with the globular head of cingulin. ZO-3 and myosin are also capable of interacting with the rest of the cingulin molecule (Cordenonsi et al., 1999). Cingulin is also able to interact with ZO-1 but this is much more complex than direct binding; rather involving multiple protein interactions (Gonzalez-Mariscal et al., 2003).

1.3.1.6 Cytoplasmic Protein: 7H6

7H6 antigen (155 kDa) plays a crucial role in the regulation of paracellular barrier function in epithelial (Zhong et al., 1993), endothelial (Satoh et al., 1996), and mesothelial (Tobioka et al., 1996) cells. 7H6 has been found to be sensitive to the TJ functional state, reversibly dissociating from the TJ in response to ATP depletion (Denker and Nigam, 1998). Reduced expression of 7H6 correlating with disruption of cellular polarity, adhesiveness and increased paracellular permeability has been found in HGF-induced cell spreading (Muto et al., 2000), liver carcinogenesis (Zhong et al., 1994), primary biliary cirrhosis (Sakisaka et al., 2001), and Helicobacter pylori exposure (Suzuki et al., 2002). During a developing TJ, 7H6 has also been found to form a continuous honeycomb linear appearance only when ZO-1 and occludin have surrounded the cellular borders, indicating 7H6 might play a role in TJ maintenance and maturation (Kimura et al., 1996; Gonzalez-Mariscal et al., 2003).

1.3.1.7 Cytoplasmic Protein: AF6

AF6 (205 kDa) is the fusion partner of ALL-1 in acute myeloid leukemia (Prasad et al., 1993). It is a multidomain protein that contains two amino-terminus Ras-binding domains, followed by kinesin and myosin like domains and a PDZ module in the middle of the protein. The carboxyl terminal contains three proline-rich domains followed by a F-actin binding region (Gonzalez-Mariscal et al., 2003). These domains allow the protein the ability to bind directly to ZO-1 at cell-cell contact sites as well as cingulin and JAM

via the PDZ domain. Ras, a small GTPase and upstream effector of the MAP kinase-signaling cascade can associate with the Ras-binding domains inhibiting the binding of ZO-1 (Yamamoto et al., 1997). This suggests a regulatory role of Ras and AF6 within TJ formation and maintenance.

1.3.1.8 Cytoplasmic Protein: Other Proteins

A wealth of information on TJ proteins has emerged in recent times and because of the focus of this dissertation, further discussion of these proteins is beyond the scope of this project. Readers are directed to the recent review paper by Gonzalez-Marsical et al. entitled "Tight junction proteins" published in *Progress in Biophysics & Molecular Biology* vol. 81, 2003, pp. 1-44, for further information.

1.3.2 Pinocytotic Activity

Pinocytosis is a process which allows for nutrient transport. It involves vesicles which are hollowed out portions of cell membranes filled with fluid forming a vacuole. However, unlike other peripheral endothelial cells BBB endothelial cell membranes have very few pinocytotic vesicles and lack fenestrations or openings (Begley, 1996). Therefore, transit across the BBB involves translocation through the capillary endothelium, internal cytoplasmic domain, and finally through the ablumenal membrane and pericyte and / or basal lamina.

1.3.3 Mitochondria

In comparison to rat peripheral endothelial cells, the BBB has a significantly greater number and volume of mitochondria (Oldendorf et al., 1977). There is an estimated five to six times more mitochondria per capillary cross-section in rat cerebral capillaries versus rat skeletal muscle capillaries (Oldendorf and Brown, 1975). This translates to increased energy potential which would be important for active transport of nutrients from the blood to the brain.

1.3.4 Enzymatic Barrier

There are numerous enzymes located at the cerebral endothelia involved in metabolizing nutrients and drugs (Minn et al., 1991; Brownlees and Williams, 1993; Brownson et al., 1994). This in effect creates an enzymatic barrier directed at metabolizing neuroactive blood-borne substances. In fact, certain enzymes such as alkaline phosphatase, aromatic acid decarboxylase, and γ-glutamyl transpeptidase are elevated in higher concentrations in cerebral microvessels versus within peripheral capillaries.

1.3.5 Cellular Wall Thickness

Brain capillaries have been found to have a decreased wall thickness of approximately 39% in comparison to muscle capillary endothelial cells (Coomber and Stewart, 1985). In addition, there was a greater concentration (seven fold) of pinocytotic vesicles in muscle capillaries as compared to cerebral capillaries. They postulate this

modulation in wall thickness maybe to allow a shortened transport time across the membrane and cytoplasm to the brain parenchyma.

1.3.6 Endothelial Cell Polarity

Quantitative biochemical studies by Betz and Goldstein in 1978 revealed a functional polarity of the BBB, meaning that there is a difference structurally and functionally between the lumenal and ablumenal surfaces of the cerebral endothelial cell (Betz and Goldstein, 1978). Enzymes such as γ-glutamyl transpeptidase and alkaline phosphatase are primarily present at the lumenal surface, however Na^{+}-K^{+}-ATPase and the sodium dependent (A-system) neutral amino acid transporter are associated with the ablumenal surface (Betz et al., 1980). The glucose receptor, GLUT-1 has also been shown to have a 3:1 ratio of distribution ablumenal to lumenal at the BBB as well (Farrell and Pardridge, 1991). The structural, biochemical, and pharmacological evidence for the polarization of enzymes, receptors, and channels within cerebral endothelia maintain the BBB is a working non-stagnant membrane evolved for maintenance of brain homeostasis (Vorbrodt et al., 1993).

1.4 Blood-Brain BarrierTransport

The purpose of the BBB is to maintain brain homeostasis, thus it must limit the exposure of the brain to potentially neurotoxic substances while at the same time allowing nutrients across. To do this, the BBB employs a number of selective transport

mechanisms including diffusion, carrier-mediated transport, and receptor-mediated, adsorptive, and fluid-phase endocytosis (Abbott and Romero, 1996; Egleton and Davis, 1997). In addition, the BBB also has efflux transporters that actively transport compounds from the brain to the blood that can limit the uptake of therapeutic compounds into the CNS (Taylor, 2002) (Figure 1.3).

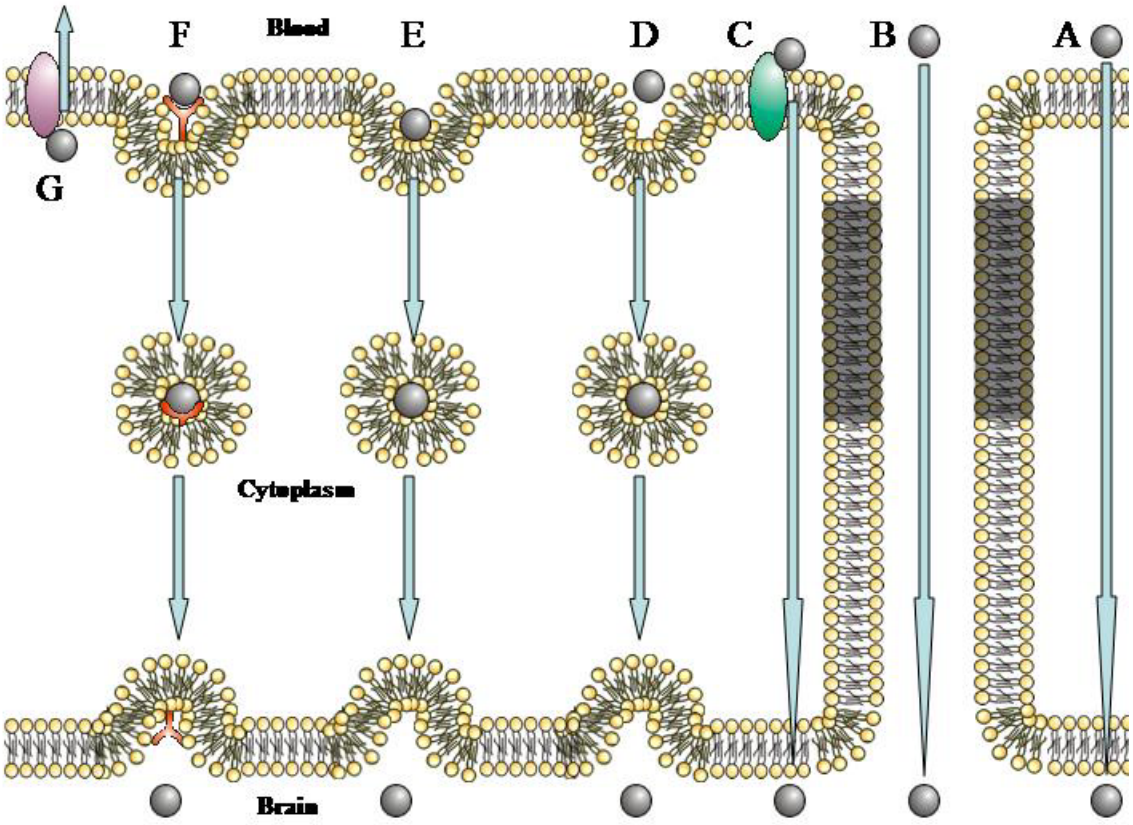

Figure 1.3 Transport mechanisms across the blood–brain barrier (BBB). (A) Transcellular diffusion; (B) paracellular diffusion; (C) carrier-mediated transport; (D) fluid-phase endocytosis; (E) adsorptive endocytosis; (F) receptor-mediated endocytosis; and (G) drug efflux. (Figure adapted from *Wolka AM, Huber JD, Davis TP. Pain and the blood-brain barrier: obstacles to drug delivery. Adv Drug Deliv Rev. 2003 Aug 28;55(8):987-1006.*)

1.4.1 Diffusion

There are four basic types of mechanisms by which most solutes traverse membranes: simple diffusion, facilitated diffusion, simple diffusion through a channel, and active transport through a protein carrier. Simple diffusion proceeds from areas of low to high concentration. Facilitated diffusion is a form of carrier-mediated endocytosis where solutes bind to specific membrane protein carriers also moving from low to high concentrations. The third type of diffusion involves an aqueous channel formed in the membrane. The active transport through a protein carrier diffusion type involves ATP hydrolysis allowing it to move against the concentration gradient.

Diffusion of substance can further be broken down into paracellular (between cells) or transcellular (across cells) diffusion which are both non-saturable and non-competitive. Because of the TJs restricting paracellular diffusion of solutes, most diffusion across the BBB is typically transcellular (Furuse et al., 1999). Thus, a solutes ability to traverse the BBB is mostly dependent on its lipophilicity and hydrogen bonding potential; the greater the lipophilicity and the lower the hydrogen bonding potential, the greater the transcellular diffusivity (Diamond and Wright, 1969; Pardridge and Mietus, 1979; Weber et al., 1991; Weber et al., 1993).

1.4.2 Carrier-mediated Transport

Carrier-mediated transport is a substrate-transporter interaction at the level of the brain endothelial surface. It is a saturable process and can be divided into a number of different mechanisms dependent on energy and/or co-transport of another substance.

Furthermore, co-transport may be in the same direction (symport) or in the opposite direction (antiport) (Egleton and Davis, 1997). Carrier-mediated transport of essential compounds has been shown with amino acids, sugars, monocarboxylic acids, and nucleosides, in and/or out of the brain. Specific transporters have been identified at the BBB for peptides such as vasopressin; neutral amino acids such as phenylalanine, leucine, and tyrosine; acidic amino acids such as glutamate and aspartate; basic amino acids such as arginine and lysine; β-amino acids such as β -alanine; monocarboxylic acids such as lactate, ketone bodies, and other short-chain fatty acids; amines such as mepyramine; the purine bases adenine and guanine; and sugars such as glucose and mannose (Tsuji and Tamai, 1999; Tamai and Tsuji, 2000).

1.4.3 Endocytosis

The BBB also employs endocytosis mechanisms for solute transport. Endocytosis can be segregated into fluid-phase and mediated endocytosis. Fluid-phase endocytosis is also known as pinocytosis which is reduced in cerebral endothelial cells as compared to the periphery. It is a nonspecific uptake of extracellular fluids at a constitutive level independent of ligand binding (Wolka et al., 2003). Fluid-phase endocytosis has been demonstrated *in vitro* in both bovine (Guillot et al., 1990) and human (Stanimirovic et al., 1996) BBB cell culture systems. Mediated endocytosis involves both receptor and absorptive mediated. Receptor-mediated endocytosis is more of a selective mode of transport. Cells have receptors for the uptake of many different types of ligands, including enzymes, hormones, plasma proteins, and growth factors. Transferrin, insulin,

and low-density lipoproteins have been demonstrated to utilize receptor-mediated endocytosis at the BBB (King and Johnson, 1985; Fishman et al., 1987; Dehouck et al., 1994; Descamps et al., 1996). Absorptive-mediated endocytosis is triggered by an electrostatic interaction between a positively charged substance (i.e. the charge moiety of a peptide) and a negatively charged plasma membrane surface (i.e. glycocalyx) (Gonatas et al., 1984). It has a lower affinity and higher capacity as compared to receptor-mediated endocytosis. Adsorptive endocytosis has been identified as the mechanism of uptake of certain cationized proteins (Kumagai et al., 1987) and peptides (Tamai et al., 1997) into the brain.

1.4.4 Efflux Transport

It has been shown that the BBB expresses a number of efflux transporters that can actively transport molecules out of the brain. Drugs, nutrients, metabolites, peptides, hormones, and neurotransmitters have all been demonstrated to be taken up by efflux transporters and moved from the brain to the blood to maintain brain homeostasis (Banks, 1999; Bart et al., 2000; Potschka et al., 2002). Efflux transporters localized at the BBB, include P-glycoprotein (P-gp), monocarboxylic acid transporters, members of the multidrug resistance-associated protein (MRP) family, and organic ion transporters (Taylor, 2002). Therapeutic compounds such as anti-HIV drugs, analgesics, antibacterials, antiepileptics, and anticancer agents into the CNS have been shown to be limited by efflux transporters (Wolka et al., 2003).

2. Pain Mechanisms

The physiologic response to pain is a very complex cascade of mechanisms involving the immune system, the CNS, and the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Wolka et al., 2003) (Figure 1.4). Numerous studies have shown these mechanisms have a significant influence upon the BBB.

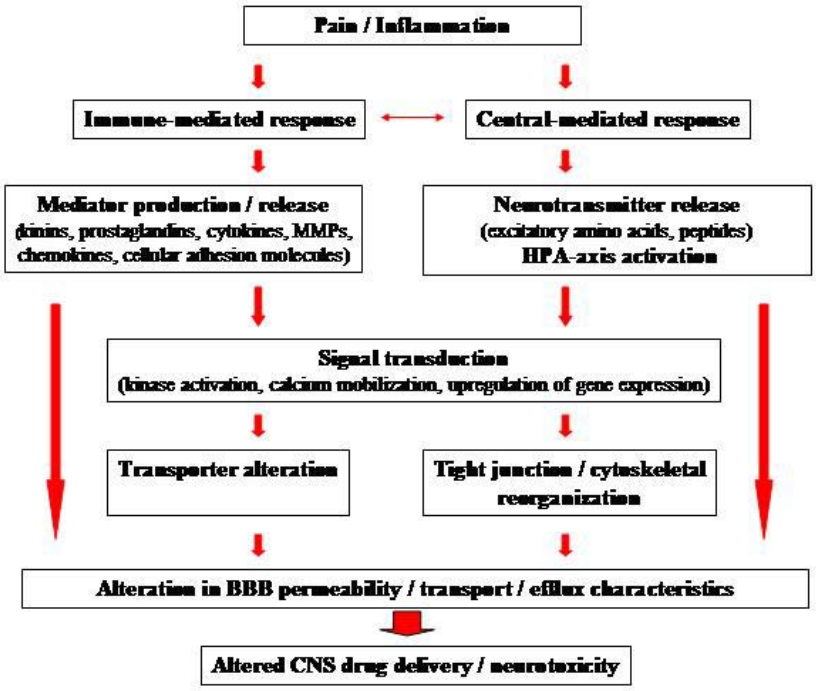

Figure 1.4 Physiological responses to pain and their potential influences on drug delivery at the BBB. (Figure adapted from *Wolka AM, Huber JD, Davis TP. Pain and the blood-brain barrier: obstacles to drug delivery. Adv Drug Deliv Rev. 2003 Aug 28;55(8):987-1006.*)

2.1 Immune and Central Nervous System

In diseases with pain/inflammation components, there exists an immune-mediated response which is characterized by the production and release of numerous inflammatory mediators. Some of these mediators include kinins, prostaglandins, cytokines, chemokines, cellular adhesion molecules and matrix metalloproteinases (MMP). They contribute to inflammation events typified by increased vascular permeability, erythema, localized edema formation, and increased leukocyte migration (Wolka et al., 2003).

The CNS response is important in pain/inflammation because neuronal pathways are involved in signaling the immune system. A precarious balance of excitatory and inhibitory neurotransmission resulting from noxious stimuli determines the intensity of nociceptive signals transmitted to the brain (Dickenson, 1999). In addition, within the CNS there is a release of proinflammatory mediators following a pain/inflammation event that can play a role in the central-mediated pain response (Wolka et al., 2003).

It is thought these inflammatory events may play a role in disrupting the interendothelial junction integrity of vessels thereby resulting in an "inflammatory permeability". Part of this may be due to cell-cell borders actively contracting and passively recoiling, resulting in larger endothelial clefts (Haselton et al., 1989; Dejana, 1997; van Nieuw Amerongen et al., 2000; Baldwin and Thurston, 2001)

2.1.1 Cytokines and Chemokines

During disease or trauma, cytokines can either be released peripherally or in the CNS. Cytokines that are thought to be involved in the immune response are the interleukins IL-1α and -1β,-2, -4, -6, -8, -10, and -13, type 1 interferons IFN-α and –β, and tumor necrosis factor-α (TNF-α) (Rivest, 2001). During inflammation both excitatory and inhibitory cytokines have been deomstrated to play a role. They have been shown to be hyperalgesic, potentiate the effect of kinins, change expression of endothelial surface molecules, and induce adhesions of polymorphoneutrophils (PMNs), monocytes, and leukocytes to cell walls (Ferreira et al., 1988; Cunha et al., 1991; Davis and Perkins, 1994; Watkins et al., 1994; Watkins et al., 1995; Fernandes et al., 2002).

In models of CNS inflammation and injury, cytokines have also been shown to disrupt the BBB. TNF-α, IL-1β, and IL-6 have been demonstrated to decrease transendothelial electrical resistance (TEER) of cultured rat cerebral endothelial cells (de Vries et al., 1996). TNF-α has also been shown to induce COX-2 expression and prostaglandin release in brain microvessel endothelial cells within the same time-frame as TNF-α induced increases in permeability and cytoskeletal structural changes. When the cells were pretreated with indomethacin, an inhibitor of COX, both the permeability and cytoskeletal changes observed with TNF-alpha were significantly reduced (Mark et al., 2001), thus suggesting cyclooxygenase activation in endothelial cells may play a role in BBB disruption. TNF-α has also been shown to downregulate occludin in astrocytes (but not in cerebral endothelial cells) and alter receptor-mediated endocytosis of low-density lipoproteins and transferrin at the BBB (Descamps et al., 1997; Wachtel et al., 2001). The cytokine IL-1α has been demonstrated to increase endothelial permeability via a

decrease in plasma membrane-associated tyrosine phosphatase activity (Gloor et al., 1997). This reduction of phosphatase activity is associated with decreased phosphorylation of tight junction proteins, decreased TEER, and loss of adherens junctions (Citi, 1992; Volberg et al., 1992; Staddon et al., 1995). IL-1β is also associated with an increase in BBB permeability and is thought to be due to a loss in occludin and ZO-1 (TJ proteins) and a redistribution of vinculin (adherens junction protein) (Bolton et al., 1998; Blamire et al., 2000).

Chemotactic cytokines or chemokines have important inflammatory, angiostatic, homeostatic, and/or angiogenic roles in disease (Godessart and Kunkel, 2001). They are expressed in both the CNS and the periphery. Chemokines have been shown to regulate cytokine production (Aliberti et al., 2000; Braun et al., 2000) and recruit leukocytes to the site of inflammation (Wolka et al., 2003). They have also been shown to modulate the BBB in a number of CNS disorders. Chemokines such as macrophage inflammatory protein 1-α and –β, monocyte chemotactic proteins (MCPs), and interferon-inducible protein 10 are produced in astrocytes, perivascular leukocytes, and microglia during inflammatory diseases such as multiple sclerosis (Bajetto et al., 2002).

2.1.2 Cellular Adhesion Molecules

Adhesion of leukocytes to the endothelial cell surface facilitates leukocyte migration across the BBB. The receptors involved in this adhesive action are classified as either selectins or integrins. Selectins are involved in the initial part of the leukocyte-endothelial cell interaction, while integrins are important for adhesion and migration

(Springer, 1995; Cid et al., 2000; Wolka et al., 2003). An immune challenge can "activate" endothelial cells resulting in an upregulation of the genes that encode for leukocyte adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Albelda et al., 1994; Wang and Feuerstein, 1995; Howard et al., 1998). Integrins are thought to play an important role during CNS inflammation because there is an increased expression during inflammatory diseases like multiple sclerosis (Sobel et al., 1998). In addition, the cytokines TNF-α, IL-1β, and IFN-γ which are known to be elevated during inflammatory CNS disease have also been shown to induce expression of ICAM-1 and VCAM-1 on brain endothelial cells and astrocytes (Merrill and Murphy, 1997).

ICAM-1 is known to be important for tethering leukocytes to the BBB (Merrill and Murphy, 1997). Increased expression of ICAM-1 is associated with a loss of occludin and ZO-1 (TJ proteins) as well as a redistribution of vinculin (adherens junction protein) resulting in increased adhesion and flux of PMNs across the BBB (Bolton et al., 1998; Edens and Parkos, 2000). An increased presence of PMNs and perivascular macrophages has also been shown to be associated with alterations in TJ proteins and modulation of the BBB (Boven et al., 2000; Edens and Parkos, 2000). The macrophage effect on the BBB is thought to contribute to HIV-1-associated dementia (Boven et al., 2000).

2.1.3 Matrix Metalloproteinases

MMPs are vascular endopeptidases important in vascular remodeling following inflammation, injury, and/or oxidative stress. They are kept in balance with tissue inhibitors of metalloproteinases (TIMPs). However, under stress MMPs are upregulated, disrupting this balance which can lead to extracellular matrix reorganization and degradation (Galis and Khatri, 2002). They have been shown to contribute to tissue damage and BBB disruption (Birkedal-Hansen, 1993; Mun-Bryce and Rosenberg, 1998; Paul et al., 1998). It has been shown that occludin is a non-matrix target of MMPs (Wachtel et al., 2001). In fact, MMP-2 which is constitutively expressed in endothelial cells near interendothelial junctions (spatially close to occludin), demonstrates how occludin could be a potential target of MMP-2 (Alexander and Elrod, 2002).

2.1.4 Kinins

Kinins are important in activating and sensitizing nociceptors, but also exert proinflammatory effects leading to edema, vasodilatation, and the release of other proinflammatory mediators (Wolka et al., 2003). Bradykinin is known to stimulate neural and neuroglial cells to produce and release prostaglandin precursors, cytokines, free radicals, and nitric oxide leading to neural cell damage and BBB disruption. (Walker et al., 1995). RMP-7, a bradykinin analogue has been shown to increase lanthanum BBB delivery *in vivo* by affecting the integrity of endothelial cell TJs (Sanovich et al., 1995). Another study demonstrated when a B_1 (kinin nociceptor) recptor agonist acted on IFN-γ upregulated B_1 receptors on human brain endothelial cells, there was a stimulation of NO

production, inhibition of IL-8 *in vitro* release, and increased BBB permeability (Prat et al., 2000).

2.1.5 Prostaglandins

PGs are released after inflammatory insult. They are produced from the action of cyclooxygenase enzymes COX-1 and COX-2 on arachidonic acid, which in turn is released from membrane phospholipids via phospholipase A_2. As discussed earlier, cytokines such as TNF-α released during inflammatory insult have been shown to induce COX-2 expression leading to increased PG production. Inflammation typically involves PGE_2 and PGE_1 (Moncada and Vane, 1982; Phipps et al., 1991). PGE_2 is known to be secreted by fibroblasts, macrophages, and malignant cells leading to vasodilation, erythema and edema formation (Harris et al., 2002). It also has been shown to control the production of cytokines and increase expression of MMPs (Hinz et al., 2000; Ikegami et al., 2001; Harris et al., 2002). PGs are also produced in BBB endothelial cells when stimulated by cytokines such as IL-1β and TNF-α (Katsuura et al., 1989; Mark et al., 2001). Thus, BBB endothelial cell PGs indirectly induces alterations in the BBB by stimulating the production of BBB altering inflammatory mediators.

2.1.6 Excitatory Peptides and Amino Acids

CNS excitatory peptides are especially important in nociception. Substance P (SP) and calcitonin gene-regulated peptide (CGRP) are two such peptides shown to affect BBB integrity. SP can stimulate the release of histamine, a proinflammatory mediator

from mast cells, leading to an increase in BBB permeability (Pernow, 1983). In addition, following stimulation by proinflammatory cytokines IL-1β and TNF-α, rat brain endothelial cells have been shown to secrete SP and bind to the cells themselves (Cioni et al., 1998). It is also thought that SP may play a role in regulation of BBB protein phosphorylation (Catalan et al., 1989).

CGRP competes for the same degradatory enzyme for SP, thereby enhancing the action of SP (Wiesenfeld-Hallin et al., 1984). Also, receptors for CGRP have been demonstrated on human brain astrocytes and cerebromicrovascular endothelial cells, indicating a possible role in BBB regulation (Moreno et al., 2002).

The amino acid glutamate (Glu) has also been shown to play a major role in BBB disruption during inflammation. Glu has been demonstrated to induce the synthesis of inflammatory mediators such as nitric oxide and platelet-activating factor (Mayhan and Didion, 1996; Nishida et al., 1996). Nitric oxide is thought to increase BBB permeability after application of Glu to cerebral endothelial cells *in vitro* (Bolton and Paul, 1997). An upregulation of Glu receptors and release of Glu by "activated" PMNs have resulted in decreased BBB function (Collard et al., 2002).

2.2 Hypothalamic-Pituitary-Adrenal Axis

The HPA axis is activated in times of stress (physical, emotional, and environmental) leading to a "stress cascade" (Miller and O'Callaghan, 2002). This activation is often due to cytokines released at the level of the CNS (Wolka et al., 2003).

Once the axis is activated, the hypothalamus releases corticotrophin-releasing hormone (CRH) stimulating the expression and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then acts on the adrenal glands causing the production and release of glucocorticoids that in turn can down regulate the immune response (Miller and O'Callaghan, 2002). These glucocorticoids or "stress hormones" modulate the expression of cytokines and adhesion molecules and feedback to inhibit the release of CRH (Elenkov and Chrousos, 2002; Webster et al., 2002).

During times of stress, neuroinflammatory disorders have been shown to worsen, such as in relapsing-remitting multiple sclerosis (Mei-Tal et al., 1970; Warren et al., 1982; Goodin et al., 1999). This process involves the release of CRH, which in turn has been hypothesized to affect the BBB directly and indirectly via mast cell activation. Through the release of TNF-α and histamine, mast cells are thought to modulate the BBB (Theoharides, 1990; Esposito et al., 2001).

3. Potential Effect of Inflammatory Pain on Blood-Brain Barrier Drug Delivery

Inflammatory pain conditions can lead to BBB dysregulation resulting in a loss of cerebral homeostasis, causing neurotoxicity and altered neuronal function (Wolka et al., 2003). As discussed previously, inflammatory pain disorders lead to a complex multitude of inflammatory mediator release and effects on the HPA axis that can create disturbances in the finely regulated BBB. Several recent studies have demonstrated and/or suggest the potential effects of inflammation on BBB permeability. In addition, it is already established that certain inflammatory pathological states have been associated with decreased BBB function such as human immunodeficiency virus-1 encephalitis (Dallasta et al., 1999), multiple sclerosis (Trojano et al., 1992), and bacterial meningitis (Kim et al., 1997).

3.1 Implications of increased paracellular blood-brain barrier permeability affecting central nervous system drug delivery

The TJs of the BBB play a role in preventing the passage of small, hydrophilic molecules from the bood to the brain (Wolka et al., 2003). However, under certain inflammatory pain states, TJ are thought to be disrupted leading to an increase in BBB permeability. This has potentially serious consequences for CNS drug delivery, as it may be increased. This can be detrimental especially if the drug has a narrow therapeutic range, but also beneficial if the drug's target is the CNS. Increased BBB drug delivery

under an inflammatory event has been shown with a platinum chemotherapeutic agent, cisplatin. Under normal circumstances, cisplatin does not cross the BBB because of its hydrophilicity (Gregg et al., 1992). However, when coadministered with lipopolysaccharide, there is an enhanced platinum content in the cerebral cortex (Minami et al., 1998). Lipopolysaccharide has been shown to decrease TEER in bovine endothelial cells (de Vries et al., 1996). It is thought that this is due to the subsequent production of inflammatory mediators. Thus, the neurotoxicity associated with cisplatin readministration (Higa et al., 1995) may be due to increased permeability from a compromised BBB (Minami et al., 1998). It is also possible that lipopolysaccharide induced inflammatory mediator release could involve TNF-α as it has been shown that TNF-α decreases TEER *in vitro* (de Vries et al., 1996) and increase cisplatin transport across bovine brain microvessel endothelial cells (Anda et al., 1997). This could prove beneficial as TNF-α could be co-administered with cisplatin to enhance its delivery across the BBB to target CNS tumors.

3.2 Implications of alterations in receptor-mediated endocytosis, glutamate release and transport, and bradykinin release

Other modes of BBB transport could potentially be affected as well. Receptor-mediated endocytosis is important in selective BBB transport of substances like transferrin, insulin, and low-density lipoprotein (King and Johnson, 1985; Fishman et al., 1987; Dehouck et al., 1994; Descamps et al., 1996). Alterations in receptor-mediated

endocytosis due to neuropathological disorders can disrupt delivery of compounds such as therapeutic drug delivery vectors, insulin, and transferrin-conjugated drugs across the BBB. Neurodegenerative disorders such as Alzheimer's and Parkinson's disease have been linked with increased levels of neuronal iron and oxidative stress (Swaiman, 1991; Jenner, 1994; Aisen et al., 1999), which could be due to disruptions in iron transport protein expression (Qian and Wang, 1998; Qian and Shen, 2001). Also, inflammatory mediators such as TNF-α have been shown to alter receptor-mediated endocytosis and transport of transferrin and low-density lipoprotein (Descamps et al., 1997).

Membrane efflux pumps at the BBB play a role in limiting drug transport into the CNS by actively transporting them out of the brain. These "pumps" include P-gp, MRP, and organic anion transporters (Wolka et al., 2003). Neurological disorders such as epilepsy and brain injury states such as ischemia/reperfusion have demonstrated an increase in efflux transporter expression (Dombrowski et al., 2001; Felix and Barrand, 2002). In addition, in other cell lines following stimulation with proinflammatory mediators, there have been alterations observed in efflux transport proteins (Piquette-Miller et al., 1998; Sukhai et al., 2001; Hartmann et al., 2002), thus indicating the possible influential effects of inflammation/pain upon BBB efflux transport.

Glutamate, important in excitatory CNS neurotransmission, also may be affected by inflammatory pain. "Activated" PMNs have been shown to release glutamate and increase expression of glutamate receptors, thereby affecting BBB transport/function (Collard et al., 2002). Thus, there is a possibility for pain-induced alterations in

glutamate transport and/or glutamate-conjugated drug delivery. This would alter the therapeutic effects of such compounds.

An analogue of the inflammatory mediator bradykinin, labradimil (cereport or RMP-7) has been used to transiently increase the permeability of the BBB and blood-brain tumor barrier. It has been shown that its mechanism of action includes disengaging the TJs of BBB endothelial cells (Emerich et al., 2001). Under times of inflammation/pain, there may be an elevation of bradykinin release affecting the BBB integrity even further. Thus, it is important to reconsider the dosing of medications like labradimil under inflammatory pain conditions (Emerich et al., 2001; Wolka et al., 2003) and understand how the endogenous release of inflammatory mediators like bradykinin might cause disruptions in BBB integrity.

4. Present Study

The blood-brain barrier (BBB) is a selective partition between the central nervous system (CNS) and peripheral circulation that limits the passage of blood-borne substances into the CNS on the basis of proteolytic stability, molecular size, charge, lipophilicity and / or solubility (Begley, 1996). The BBB is characterized by tight junctions (TJs) between brain capillary endothelial cells and a lack of fenestrations, characteristics which help the BBB maintain brain homeostasis. The TJs, which severely restrict paracellular diffusion of solutes into the brain, are composed of a network of several transmembrane and cytoplasmic proteins linked to an actin cytoskeleton (Reese and Karnovsky, 1967; Drewes, 2001; Huber et al., 2001). BBB dysregulation can result in loss of maintenance of brain homeostasis, resulting in altered neuronal function and potential neurotoxicity (Wolka et al., 2003). Pathological states such as human immunodeficiency virus-1 encephalitis (Dallasta et al., 1999), multiple sclerosis (Trojano et al., 1992), non-occlusive hypoxia (Witt et al., 2003), Alzheimer's disease (Kalaria, 1996), cerebral malaria (Brown et al., 2001), and bacterial meningitis (Kim et al., 1997) have all been associated with decreased BBB function. Recent studies indicate inflammation also changes BBB structure and permeability (Wolka et al., 2003). These alterations can lead to nutritional and ionic imbalances in the CNS, resulting in dysregulation of neuronal function, and altered delivery of therapeutic agents potentially causing improper dosing regimens leading to neurotoxicity.

4.1 Specific Aims

Currently much research has been done on investigating drug delivery and the BBB under a naïve state; this dissertation examines the BBB under a pathophysiological state and what that means clinically. This dissertation focuses on investigating the effect of peripheral inflammatory pain on the BBB in terms of molecular changes and therapeutic drug delivery, as altered delivery of therapeutic agents may lead to incorrect dosing and neurotoxicity. Using a BBB membrane impermeant marker, sucrose and codeine, an analgesic drug commonly used for the treatment of inflammatory pain this examination will assess the following specific aims.

1. Assess the BBB permeability of [^{14}C] sucrose and [^{3}H] codeine under three inflammatory pain models: formalin, λ-carrageenan, and complete Freund's adjuvant. *(Chapter 2)*
2. Determine the functional effect of inflammatory pain on antinociception of codeine. *(Chapter 3)*
3. Examine the viability of microvascular endothelial cells to direct exposure of formalin, λ-carrageenan, and complete Freund's adjuvant. *(Chapter 4)*
4. Investigate the molecular effects of inflammatory pain on BBB tight junction proteins. *(Chapter 5)*
5. Explain the overall influence of inflammatory pain on the BBB. *(Chapter 6)*

4.2 Overlying Hypothesis

Inflammatory pain increases the BBB permeability of sucrose and codeine via paracellular diffusion by affecting the regulation and expression of tight junction proteins.

CHAPTER 2. THE EFFECT OF INFLAMMATORY PAIN ON BLOOD-BRAIN BARRIER DRUG DELIVERY

Chapter studies published in:

Hau, V.S., Huber, J., Campos, C. and Davis, T.P.: Effect of λ-carrageenan induced inflammatory pain on codeine blood-brain barrier permeability and antinociception. Brain Research **1018(2)**: 257-64, 2004.

Huber, J.D., **Hau, V.S.**, Campos, C.R., Egleton, R.D., and Davis, T.P.: Functional and structural analysis of blood-brain barrier tight junctions during a 72 hr exposure to λ-carrageenan induced inflammatory pain. Am J Physiol Heart Circ Physiol **283 (4)**: H1531-7, 2002.

Introduction

These initial studies focused on the effects of peripheral inflammatory pain on BBB drug delivery. Currently, little is known about the influence of peripheral inflammation on the BBB. To investigate this further we used three well-characterized and established inflammatory pain models in the rat: formalin, λ-carrageenan, and complete Freund's adjuvant (CFA). Each of these models is characterized by a different onset and time course of inflammatory response. With each model we induced inflammation by unilateral subcutaneous injection of the right hind paw with the respective inflammatory agent. We then compared each pain model to determine whether peripheral inflammation had an effect on functional BBB permeability using the *in situ* brain perfusion technique with a BBB impermeant marker, sucrose.

Once, it was established that peripheral inflammation did have an effect on BBB permeability, we investigated the effects of λ-carrageenan-induced inflammatory pain over a time course (0-168 h) of inflammation. The λ-carrageenan-induced pain model was chosen due to its onset of action and duration of effects compared with both the formalin and CFA-induced pain models. In addition to sucrose, a common clinically used drug for inflammatory pain, codeine was investigated to demonstrate how peripheral inflammation may affect drug delivery clinically.

Methods

Radioisotopes and Chemicals

[^{14}C] sucrose was obtained from ICN Pharmaceuticals (specific activity, 492 mCi/mmol, >99.5% purity; Irvine, CA). [^{3}H] codeine was obtained from American Radiolabeled Chemicals, Inc. (specific activity, 80 Ci/mmol, 99% purity; St. Louis, MO). All other chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO).

Animals and Treatments

Female Sprague-Dawley rats (250-300 g; Harlan Sprague Dawley, Indianapolis, IN) were housed under standard 12:12-h light-dark conditions and received food ad libitum. Protocols used in this study were approved by the University of Arizona Institutional Animal Care and Use Committee and abided by NIH guidelines. To induce inflammatory pain, rats were briefly anesthetized under 5% halothane gas. In the multiple inflammatory pain model study, either 0.9% saline control, 5% formalin, 3% λ-carrageenan or 50% CFA (100 uL; s.c.) was injected into the plantar surface of the right hind paw. For the λ-carrageenan time course study either 3% λ-carrageenan or 0.9% saline was injected in a similar fashion.

In situ Brain Perfusion

Immediately prior to *in situ* brain perfusion, rats were anesthetized with sodium pentobarbital (60 mg/kg; i.p.). At 1-h postinjection, the 0.9% saline control and 5%

formalin-injected rats underwent a 20-min in situ perfusion. λ-carrageenan (3%)- and CFA (50%)-injected rats underwent perfusion at 3 h and 3 days, respectively. In the λ-carrageenan time course study, at time points ranging from 0 (saline control) to 168 h post-injection of λ-carrageenan, the rats underwent a 20 min *in situ* brain perfusion (Figure 2.1) of radiolabeled compound ([^{14}C] sucrose or [^{3}H] codeine). Naïve controls (no paw injection) showed no significant difference in BBB permeability to sucrose compared to saline-injected controls and therefore these data are not shown. *In situ* brain perfusion studies were carried out based upon the methods of Preston (Preston et al., 1995). Briefly, rats were anesthetized with sodium pentobarbital as described above and heparinized (10,000 U/kg). A constant body temperature was maintained using a heating pad. The common carotid arteries were cannulated with silicone tubing connected to a perfusion circuit. The perfusate was an erythrocyte-free modified mammalian Ringer's solution made of (in mM) 117 NaCl, 4.7 KCl, 0.8 $MgSO_4$, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, and 10 D-glucose, along with 3.9% dextran (molecular weight 70,000) and 1 g/L bovine serum albumin (type V). A control for BBB integrity was provided by the addition of Evans blue (55 mg/L) to the Ringer's solution. The perfusate was warmed to 37°C and aerated with 95% O_2-5% CO_2. To allow drainage, the jugular veins were sectioned. Once the desired perfusion pressure of 85-95 mm/Hg and flow rate of 3.1 mL/min were achieved, either [^{14}C] sucrose or [^{3}H] codeine was infused using a slow-drive syringe pump (0.5 mL/min per hemisphere; model 22, Harvard Apparatus, South Natick, MA) into the perfusate inflow. After a 20 min perfusion, the animal was decapitated and the brain removed. The choroid plexuses and meninges were excised,

and cerebral hemispheres sectioned and homogenized. At termination of the perfusion, perfusate containing the radiolabeled marker was collected from each carotid cannula to serve as a reference.

One mL of tissue solubilizer (TS-2; Research Products, Mount Pleasant, IL) was added to cerebral hemispheres (~500 mg) and perfusate (100 µL) reference. Samples were solubilized for 2 d, after which 100 µL of 30% glacial acetic acid was added to eliminate chemiluminescence. Four mL of Budget Solve Liquid Scintillation Cocktail (Research Products) were added, and samples were measured for radioactivity on a liquid scintillation counter (model LS 5000 TD Counter; Beckman Instruments, Fullerton, CA).

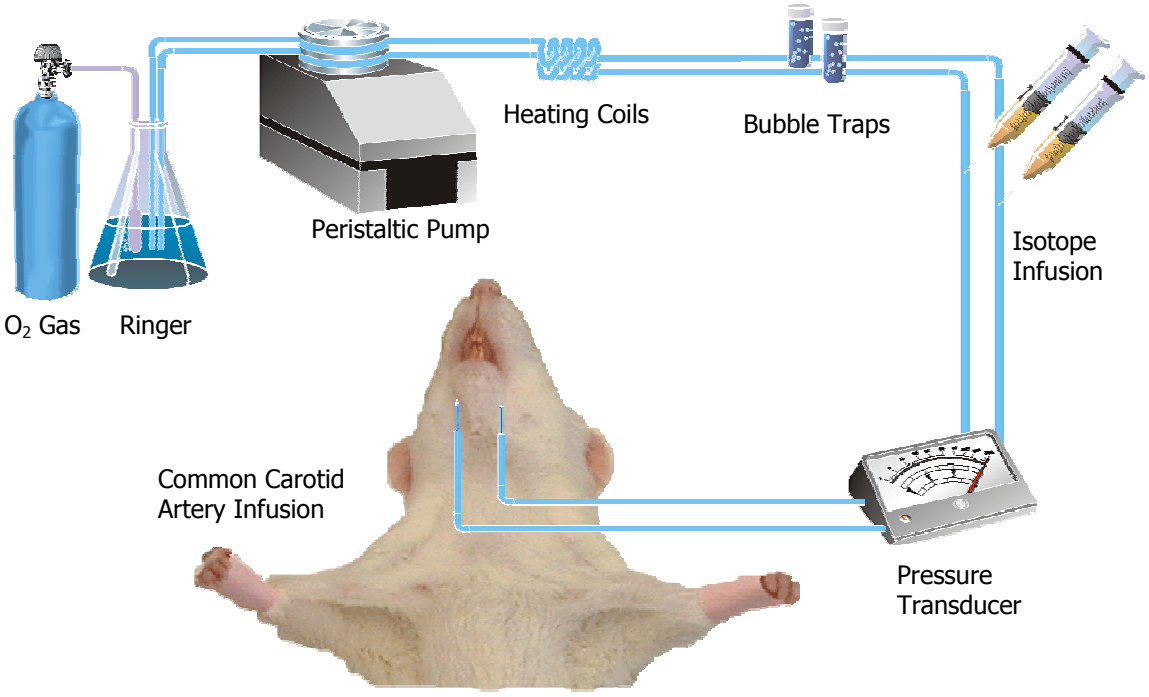

Figure 2.1 A schematic diagram of the *in situ* brain perfusion technique. Both common carotid arteries are cannulated and brain perfused with an oxygenated mammalian Ringer. Radiolabelled compounds are studied by infusing the lines via a slow drive syringe pump. Jugular veins are sectioned for perfusate outflow.

Capillary Depletion

Measurement of the vascular component to total brain uptake was performed using a capillary depletion method by Triguero (Triguero et al., 1990). Post 20 min *in situ* perfusion, the brain was removed, and choroid plexuses and meninges were excised. Brain tissue (50 mg wet weight) was placed in 1.5 mL of capillary depletion buffer [containing (in mM) 10 4-(2-hydroxyethyl)-piperaxineethane sulfonic acid, 141 NaCl, 4 KCl, 2.8 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, and 10 D-glucose; pH 7.4] and homogenized (Polytron homogenizer, Brinkman Instruments, Westbury NY) on ice. Homogenization was repeated with ice-cold 26% clinical-grade dextran (2 mL). To separate the vascular component from the homogenate, aliquots of homogenate were centrifuged at 5,400 x *g* for 15 min. The vascular pellet was separated from the capillary-depleted supernatant. All homogenization procedures were performed within 2 min of euthanizing the animal. All components of the sample (homogenate, supernatant, and pellet) were taken for radioactive counting.

Calculation of In situ Brain Perfusion and Capillary Depletion

Results were expressed as ratio of brain tissue to perfusate activities (R_{brain}), which is equal to the total amount of radiolabeled isotope in the whole brain, brain homogenate, supernatant, or pellet (C_{brain}; in disintegrations $\cdot$ min $^{-1}$ $\cdot$ g $^{-1}$ or disintegrations $\cdot$ min $^{-1}$ $\cdot$ mL $^{-1}$) divided by amount of radiolabeled isotope in perfusate ($C_{perfusate}$; in disintegrations $\cdot$ min $^{-1}$ $\cdot$ mL $^{-1}$).

$$\mathbf{R_{brain}\% = (C_{brain}/\ C_{perfusate})\ x\ 100\%}$$

Cerebral Blood Flow

The perfusion method of Preston et al. (Preston et al., 1995) and Zlokovic et al. (Zlokovic et al., 1986) was adapted to determine both CBF and the rate of cerebral perfusion *in situ* using the derived equations of Gjedde et al. (Gjedde et al., 1980) for [^{3}H] butanol uptake. *In situ* brain perfusion was carried out as stated above with a Ringers solution containing 4 ml/l unlabeled ethanol. With the use of a slow-drive syringe pump (0.5 ml/min per hemisphere), [^{3}H] butanol was added during last 10 s of a 20-min perfusion. A partition coefficient (λ_r) was determined using a separate group of animals perfused with a constant [^{3}H] butanol concentration in the arterial inflow for 20 min followed by brain sampling and analysis. Brains were immediately weighed and sectioned. Brain and Ringer solution samples were taken for liquid scintillation counting. A small portion of the frontal lobes (~50 mg) was removed and weighed separately to determine the brain tissue dry weight by drying in an oven at 95°C to constant weight. Unlabeled ethanol was added to saturate endogenous alcohol dehydrogenase for both measurements.

Calculation of Cerebral Blood Flow

The basic treatment of Gjedde et al. (Gjedde et al., 1980) was followed using the derived equation

$$\mathbf{F_{bl} = -\lambda_{br}\ ln[(1 - C_{br(t)} / \lambda_{br}\ x\ C_a) / t]}$$

where F_{bl} is the rate of blood flow [in ml/min per unit mass (g)] and C_a is the constant [^{3}H] butanol concentration in arterial inflow at *time t* between the introduction of [^{3}H] butanol and decapitation. C_{br} is the activity in unit weight of brain at *time t*. λ_{br} is the distribution ratio of [^{3}H] butanol between the brain and the perfusion medium at the steady state. The value of λ_{br} was calculated as the ratio of the [^{3}H] radioactivity in the brain versus [^{3}H] radioactivity in the arterial inflow. Extraction of the tracer from the blood is assumed to be complete during a single capillary pass.

Data Analysis

In all experiments, data were presented as mean ± SE. To determine significance of changes in BBB permeability to [^{14}C] sucrose or [^{3}H] codeine, a one-way analysis of variance (ANOVA) was performed on calculated R_{brain}% values at each time point. Capillary depletion data were analyzed using a two-way ANOVA to determine significance between time points, fractions, and interaction between (time x fraction). Significance was defined as $p<0.05$. All analyses were performed using the Sigma Stat 2.03 statistical software package (SPSS Inc.; San Rafael, CA).

Results

BBB Permeability of [^{3}H] Codeine with Formalin-, λ-Carrageenan-, and CFA- Induced Inflammatory Pain Models

Assessment of formalin-, λ-carrageenan-, and CFA-induced inflammatory pain effects on BBB permeability was done at the respective point of maximal inflammation for each model. This was investigated with *in situ* perfusion of the brain with [^{14}C] sucrose, a membrane-impermeant marker. Post-*in situ* perfusion brain parenchymas were examined for gross breakdown of the BBB indicated by presence of Evans blue albumin influx. Only brains with morphologically intact BBB were analyzed.

Figure 2.2 shows the control group, representing the vascular space volume, in comparison to the three inflammatory pain models. Each bar (mean ± SE (n = 7-8)) represents a ratio of the radioactivity found in the brain to radioactivity found in the perfusate media (R_{brain}%). The control group had a R_{brain}% value of 1.71 ± 0.09% which when converted to a vascular space of 17.1 μl/g brain tissue is consistent with previous studies using vascular markers (3-20 μL/g brain) (Heisey, 1968; Blasberg et al., 1983; Williams et al., 1996). The results showed all three inflammatory pain models compared to control had a significantly (p<0.01) higher distribution of sucrose into the brain (formalin, 67.3%; λ-carrageenan, 62.5%; and CFA, 87.6%).

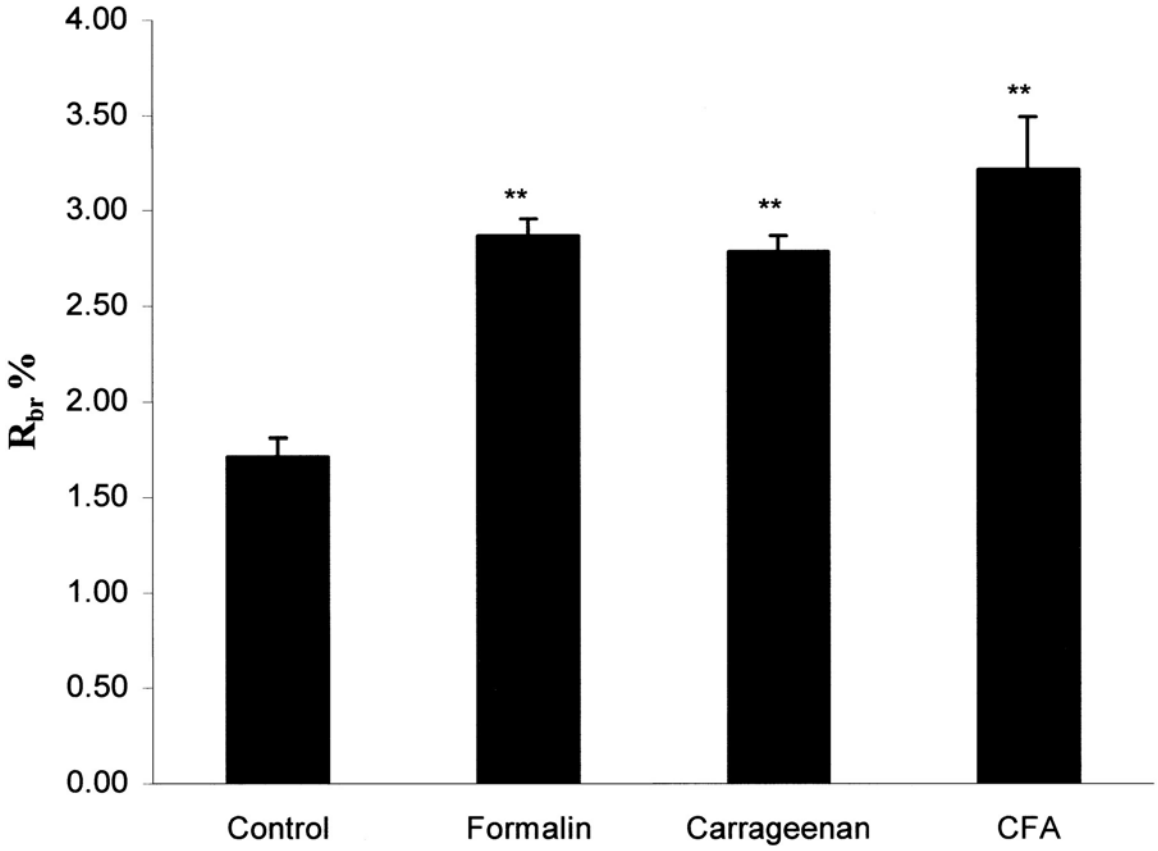

Figure 2.2 [^{14}C] sucrose BBB permeability post 20-min *in situ* perfusion after right hind paw injection with saline, formalin, λ-carrageenan or CFA. Results indicate a significantly higher distribution of sucrose in the cerebral hemispheres for all inflammatory pain models at their time points of maximal inflammation [formalin (1h), 67.3%; λ-carrageenan (3h), 62.5%; and CFA (72h), 87.6%] compared with that in control. R_{br}, ratio of radioactivity found in the brain compared with the radioactivity found in the perfusate media. Each bar represents the mean ± SE (n = 7-8). Statistical significance was determined using one-way ANOVA followed by Newman-Keuls post hoc test. $^{**}P < 0.01$ vs. control.

Table 2.1 compares capillary depletion data post 20-min *in situ* perfusion of all three inflammatory pain models with control. Results showed the amount of [^{14}C] sucrose in all three inflammatory pain model pellets was not significantly (p<0.05) different from that in control. In addition, the actual entry of [^{14}C] sucrose into the brain

parenchyma as measured by percent amount of [^{14}C] sucrose associated with the supernatant was not significantly (p<0.05) different from the homogenate.

Table 2.1 Capillary depletion studies after a 20-min *in situ* perfusion

Model	Pellet	Supernatant	Homogenate
Control	0.69 ± 0.17†	1.71 ± 0.09	1.86 ± 0.11
Formalin	0.79 ± 0.17†	1.94 ± 0.10	2.31 ± 0.12*
λ-Carrageenan	0.92 ± 0.15†	2.48 ± 0.18*	2.92 ± 0.17*
CFA	1.16 ± 0.25†	3.19 ± 0.30*	3.13 ± 0.14*

Values are means ± SE; n = 4. Data are the percent values after a 20-min *in situ* perfusion with [^{14}C] sucrose. CFA, complete Freund's adjuvant. Statistical significance was determined using 2-way ANOVA. * $P < 0.01$, significant difference from control within group (pellet, supernatant, or homogenate); $^{\dagger}P < 0.01$, significant difference from homogenate.

Table 2.2 shows CBF parameters post 20 min *in situ* perfusion. There was no significant difference among the three pain models in cerebral perfusion pressures and rates when compared to control. CBF (F_{bl}) was calculated at t = 10 s: $C_{br(t)}$ = control, 0.76 ± 0.07 μCi/g; formalin, 0.98 ± 0.12 μCi/g; λ-carrageenan, 1.16 ± 0.20 μCi/g; and CFA, 1.07 ± 0.20 μCi/g; C_a = control, 34.11 ± 0.75 nCi/g; formalin, 33.07 ± 1.88 nCi/g; λ-carrageenan, 38.06 ± 1.05 nCi/g; and CFA, 37.62 ± 1.77 nCi/g; and λ_{br} = control, 0.61; formalin, 0.64; λ-carrageenan, 0.80; and CFA, 0.52. There was a significant ($P < 0.01$) increase in CBF in all three pain models compared to control (0.82 ± 0.01 ml·min^{-1}·g^{-1}). Perfused brain weight and water content were not significantly different from control.

Table 2.2 Cerebral blood flow analyses after a 20-min *in situ* perfusion

Treatment	Control	Formalin	λ-Carrageenan	CFA
λ_{br}	0.61 ± 0.06 (3)	0.64 ± 0.05 (3)	0.80 ± 0.10 (3)	0.59 ± 0.05 (3)
Perfusion pressure, mmHg	93.61 ± 3.55 (9)	83.68 ± 3.49 (8)	80.15 ± 3.59 (8)	82.98 ± 3.11 (8)
Perfusion rate, $ml \cdot min^{-1} \cdot g^{-1}$	1.70 ± 0.019 (5)	1.66 ± 0.042 (4)	1.71 ± 0.015 (4)	1.72 ± 0.007 (4)
Cerebral blood flow, $ml \cdot min^{-1} \cdot g^{-1}$	0.82 ± 0.01 (5)	0.94 ± 0.01† (4)	1.41 ± 0.03† (4)	0.89 ± 0.02* (4)
Perfused brain weight, g	1.82 ± 0.02 (5)	1.87 ± 0.046 (5)	1.81 ± 0.016 (5)	1.80 ± 0.008 (5)
Perfused brain water, %	82.65 ± 0.11 (5)	82.57 ± 1.14 (5)	82.108 ± 0.72 (5)	84.47 ± 1.00 (5)

Values are means ± SE; numbers in parentheses are numbers of rats. λ_{br}, Distribution ratio of [^{3}H] butanol between the brain and perfusion medium at the steady state. Significance was determined using one-way ANOVA followed by a Newman-Keuls post hoc test. * $P < 0.05$, † $P < 0.01$ vs. control.

0 - 168 h BBB Permeability Time Course of [^{14}C] Sucrose under λ-Carrageenan-Induced Inflammatory Pain

The effect of λ-carrageenan-induced inflammatory pain on BBB permeability over a 0 to 168 h time course was assessed using [^{14}C] sucrose. Gross assessment of brain parenchyma post-*in situ* perfusion showed no Evans blue albumin influx, indicating the BBB was morphologically intact.

Figure 2.3 shows R_{brain}% values from 0 to 168 h following λ-carrageenan treatment. Bars represent mean ± SE (n = 6). R_{brain}% value for saline control (0 h) rats was 1.8 ± 0.2%. This value is representative of vascular volume and can be converted to a vascular space of 18 μL/g brain tissue, which is consistent with the multiple

inflammatory pain model study discussed above. Results showed a biphasic response with a significantly (p<0.05) higher distribution of sucrose associated with the brain at 1, 3, 6, and 48 h (139 ± 9%, 166 ± 19%, 138 ± 13%, and 146 ± 7% compared with control, respectively). [^{14}C] Sucrose permeability returned to control levels at 12, 24 and 72 h and baseline permeability was still observed at 168 h.

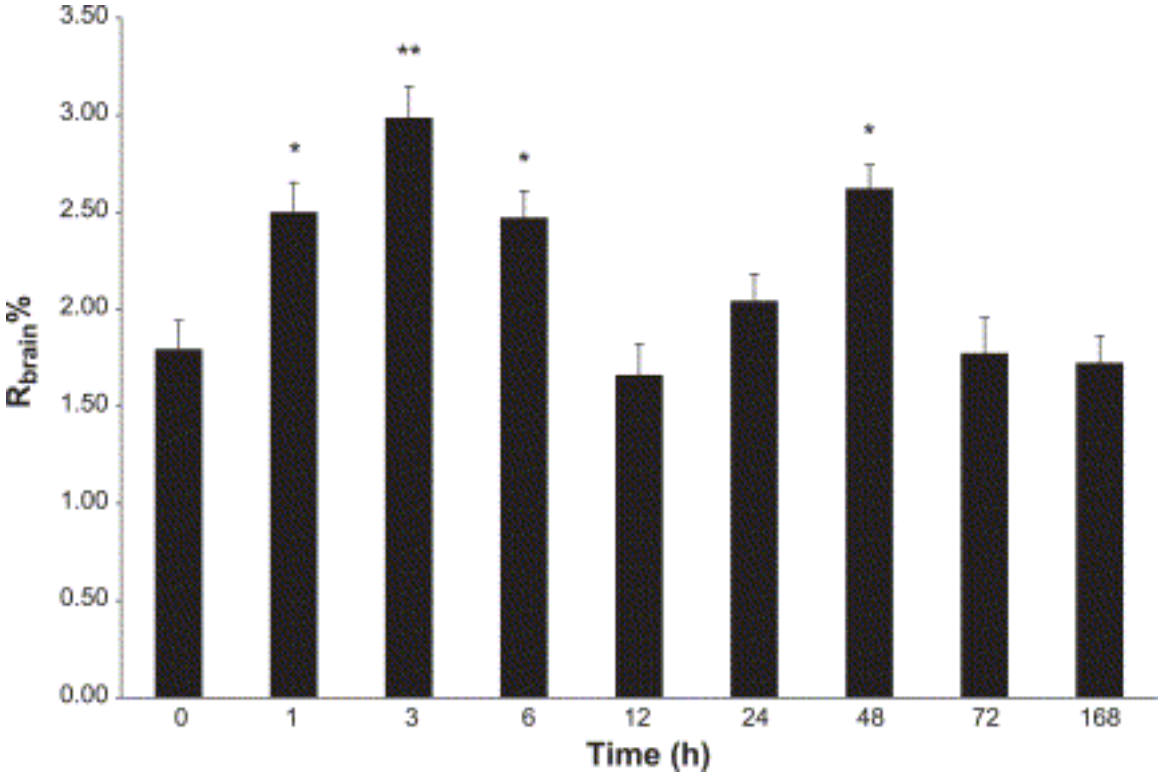

Figure 2.3 BBB permeability time course of [^{14}C] sucrose after λ-carrageenan-induced inflammatory pain. Bars represent mean±S.E. (n=6). %R_{brain} represents percent radioactivity found in brain parenchyma compared with perfusate radioactivity after 20 min *in situ* brain perfusion with [^{14}C] sucrose. Results demonstrate a significant increase in BBB permeability at 1, 3, 6 and 48 h versus 0 h (saline control). Statistical analysis was done using a one-way ANOVA followed by Tukey's HSD post-hoc analysis. *p<0.05 and **p<0.01 versus control.

Capillary depletion results after *in situ* brain perfusions demonstrated no difference compared to control in the amount of [^{14}C] sucrose associated with the pellet at various time points (Table 2.3). In addition, there were significantly (p<0.05) lower amounts of radioactivity associated with the pellets as compared to brain homogenates. The amount of [^{14}C] sucrose associated with brain supernatants were not statistically different (p>0.05) from amounts associated with homogenates.

Table 2.3 Capillary depletion studies after a 20-min *in situ* brain perfusion

Time (h)	Pellet	Supernatant	Homogenate
Control	0.3 ± 0.1*	1.8 ± 0.4	1.9 ± 0.2
1 h	0.3 ± 0.1*	2.8 ± 0.2	2.7 ± 0.3
3 h	0.2 ± 0.2*	3.1 ± 0.3	2.9 ± 0.3
6 h	0.3 ± 0.1*	2.5 ± 0.1	2.6 ± 0.2
12 h	0.3 ± 0.2*	1.5 ± 0.2	1.7 ± 0.2
24 h	0.3 ± 0.1*	1.8 ± 0.1	1.8 ± 0.1
48 h	0.2 ± 0.1*	2.3 ± 0.3	2.7 ± 0.4
72 h	0.3 ± 0.1*	2.0 ± 0.2	1.7 ± 0.5

Values are means ± SE; n = 5. Data are the percent values after a 20-min *in situ* brain perfusion with [^{14}C] sucrose. Statistical significance was determined using two-way ANOVA, followed by Tukey's honestly significant difference post hoc analysis. * P < 0.01, significant difference from homogenate within a treatment group. No significant difference was determined between treatment groups, and no significant interaction (time point × fraction) was observed (P > 0.05).

0 - 168 h BBB Permeability Time Course of [^{3}H] Codeine under λ-Carrageenan-Induced Inflammatory Pain

The effect of λ-carrageenan-induced inflammatory pain on BBB permeability was assessed over a 0 to 168 h time course using [^{3}H] codeine. Because the greatest changes

in sucrose permeability were observed at 3 and 48 h, codeine uptake was studied at these time points. Additionally, codeine uptake was investigated at 0 h (saline control) and 168 h.

Figure 2.4 shows R_{brain}% for codeine at the time points 0, 3, 48 and 168 h following λ-carrageenan treatment. Bars represent mean ± SE (n = 4). Results demonstrated a significantly (p<0.01) higher distribution of [^{3}H] codeine associated with the brain at 3 and 48 h (179 ± 6% and 179 ± 12% compared with saline control, respectively).

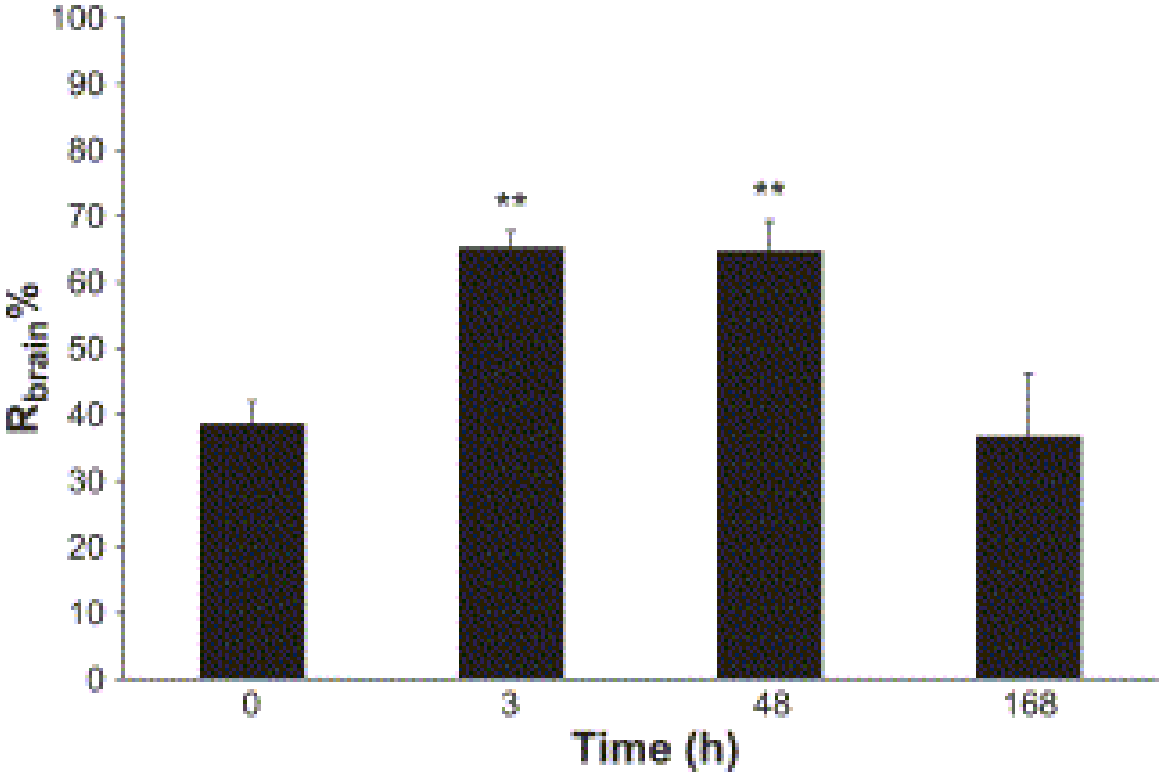

Figure 2.4 BBB permeability time course of [^{3}H] codeine after λ-carrageenan-induced inflammatory pain. Bars represent mean±S.E. (n=4). %R_{brain} represents percent radioactivity found in brain parenchyma compared with perfusate media radioactivity after 20 min *in situ* brain perfusion with [^{3}H] codeine. Results demonstrate a significant increase in BBB permeability at 3 and 48 h versus 0 h (saline control). Statistical analysis was done using a one-way ANOVA followed by Tukey's HSD post-hoc analysis. *p<0.05 and **p<0.01 versus control.

Capillary depletion data after *in situ* brain perfusions showed the amount of [^{3}H] codeine associated with the pellet at various time points was not significantly (p>0.05) different from control (0 h) (Table 2.4). In addition, there were significantly (p<0.05) lower amounts of radioactivity associated with the pellets as compared to brain

homogenates, and the amounts of [^{3}H] codeine associated with brain supernatants were not statistically different (p>0.05) from the amounts associated with homogenates.

Table 2.4 [^{3}H] Codeine capillary depletion values after 20 min *in situ* brain perfusion

Time (h)	Pellet	Supernatant	Homogenate
Control (0)	7.1 ± 0.8†	35.2 ± 3.6	31.4 ± 4.0
3	10.0 ± 1.2†	49.9 ± 4.4	46.3 ± 3.8
48	8.5 ± 1.0†	42.0 ± 4.9	41.9 ± 4.7
168	9.8 ± 2.4†	28.0 ± 4.6	27.3 ± 3.3

Values represent mean±S.E. (n=4). Values are %R_{brain} of fractions after 20 min *in situ* brain perfusion with [^{3}H] codeine. Statistical analysis was done using a two-way ANOVA, followed by Tukey's HSD post-hoc analysis.

Discussion

BBB Permeability of [^{3}H] Codeine with Formalin-, λ-Carrageenan-, and CFA- Induced Inflammatory Pain Models

Three different inflammatory pain models (acute, short term, and long term) were used to investigate *in vivo* BBB permeability changes after peripheral inflammation stimuli. Using *in situ* brain perfusion with [^{14}C] sucrose, the effect of inflammation on basal permeability across an intact BBB was assessed. The control basal permeability as measured by R_{brain}% value was 1.71% which when converted to a vascular space of 17.1 µl/g brain tissue is similar to that found in other studies (Heisey, 1968; Blasberg et al., 1983; Williams et al., 1996) using vascular markers (3-20 µl/g brain). Studies of the three inflammatory pain models showed significant increases in R_{brain}%, indicating a change in vascular volume and/or an increase in BBB permeability (Fig. 2.2).

To elucidate the cause of these permeability increases, capillary depletion studies were performed. Capillary depletion studies can help determine whether the increased BBB permeabilities were due to increased paracellular diffusion and not changes in vascular space or vascular trapping. Results (Table 2.1) showed the amount of sucrose trapped in the vascular pellet of each of the three pain models was not statistically different from control. However, there was a significant ($P < 0.01$) increased sucrose associated with the supernatant in the λ-carrageenan and CFA groups indicating a breach in the BBB. Furthermore, the percent amount of [^{14}C] sucrose associated with actual

entry into the brain parenchyma (supernatant) was not significantly different from the homogenate, indicating the isotope was of parenchyma origin versus vascular trapping.

Cerebral autoregulation is important for protecting the brain from fluctuations in flow and pressure in the peripheral circulation to maintain a constant brain perfusion. Therefore, cerebral blood flow dynamics should be considered because of potential alterations in cerebral perfusion causing an over- or underestimation of permeability. CBF [^{3}H] butanol values measured in control rats (0.82 ± 0.01 ml·min^{-1}·g^{-1}) were similar to previously reported values ranging from 0.8-1.49 ml·min^{-1}·g^{-1} (Gjedde et al., 1980; Sage et al., 1981). When compared to all three inflammatory pain models, there was a significant ($P < 0.01$) increase in CBF within the models (Table 2.2). This also corresponded with a nonsignificant increase in capillary depletion (Table 2.1) suggesting a possible slight increase in vascular volume. Also, increases in CBF were only accompanied by a small drop in perfusion pressure and a constant perfusion rate, indicating maintenance of cerebral autoregulation during inflammation. In addition, the nonsignificant difference in percent brain water content shows edema formation was negligible. This CBF data indicates that inflammatory pain causes significant increases in BBB permeability via increased paracellular diffusion.

These initial studies demonstrated inflammatory-mediated pain states alter the functional properties of the BBB. Capillary depletion and cerebral blood flow studies further elucidated the cause was due to alterations in paracellular diffusion rather than vascular trapping or space. Also, because [^{14}C] sucrose is a membrane impermeant marker, paracellular diffusion is its primary mode of delivery across the BBB. Therefore,

increases in [^{14}C] sucrose permeability are most likely due to alterations in BBB endothelial cell tight junctions leading to an increase in paracellular diffusion.

0 - 168 h BBB Permeability Time Course of [^{14}C] Sucrose under λ-Carrageenan-Induced Inflammatory Pain

Now that it was determined peripheral inflammatory-mediated pain states increased BBB permeability and altered paracellular diffusion at peak inflammation, we wanted to further study the effects over a time course. With a time course study we could better determine what regulatory effects were occurring to the BBB manifested by functional changes in BBB permeability. We investigated the effects of λ-carrageenan-induced inflammatory pain on the functional integrity of BBB tight junctions over the time course (0-72 h) of inflammation. We extended the [^{14}C] sucrose BBB permeability time course to 168 h from our previous inflammatory pain model studies to ensure an end point at which BBB permeability returned to basal levels (t = 0). The λ-carrageenan-induced pain model was chosen due to its onset of action and duration of effects compared with both the formalin and CFA-induced pain models. The effects of λ-carrageenan-induced inflammation on basal permeability across an intact BBB were again assessed using *in situ* brain perfusion with [^{14}C] sucrose. The control vascular space volume of 17.9 μl/g brain tissue was similar to that in our previous study (17.1 μl/g brain tissue) and consistent with other studies using vascular space markers (Heisey, 1968; Blasberg et al., 1983; Williams et al., 1996). λ-Carrageenan-induced inflammation elicited a biphasic increase in BBB permeability at 1-6 h and at 48 h (Figure 2.3). The

increase seen in this study from 1 to 6 h was consistent with our previous findings at 3 h and demonstrated the maximal increase in BBB permeability coincided with maximal inflammatory response.

Previous studies (Lossinsky et al., 1983; Preston and Foster, 1997; d'Avella et al., 1998) investigating changes in BBB permeability have found evidence of increased vesicular transport. Therefore, to investigate the possible contribution of increased vesicular activity to the increased association of sucrose with the brain, capillary depletion studies were again conducted (Table 2.3). These studies showed the amount of sucrose trapped in the vascular pellet at each time point was not statistically different from the control and the amount of radioactivity associated within the pellet was not significantly different among time points compared with the control. These results suggest the increased BBB permeability observed in this study was not due to changes in vascular volume (i.e., changes in cerebral blood flow, vasoconstriction/dilatation) or increased vascular trapping (i.e., increased endocytotic activity), thereby indicating that λ-carrageenan-induced inflammatory pain significantly increased BBB permeability, most likely via increased paracellular diffusion between brain microvascular endothelial cells as suggested previously by the multiple inflammatory pain model study.

Although the increase in BBB permeability was relatively small (~66% increase over the 20-min period at 3 h) in magnitude, the implications of these increases are physiologically significant. Generally, we would not expect a several-fold increase in BBB permeability, as seen after osmotic disruption (~300% increase in BBB permeability after 1.6 mM mannitol infusion), following a peripheral inflammatory insult. If this were

the case, the BBB would become compromised after every inflammation or infection. Rather, our primary concern centers on disruption of CNS homeostasis and proper neuronal function. Several CNS pathologies, including human immunodeficiency virus-1 encephalitis (Dallasta et al., 1999), multiple sclerosis (Trojano et al., 1992), non-occlusive hypoxia (Witt et al., 2003), Alzheimer's disease (Kalaria, 1996), cerebral malaria (Brown et al., 2001), and bacterial meningitis (Kim et al., 1997), have shown a correlation between increased BBB permeability and altered CNS homeostasis and neuronal function. Furthermore, there are numerous therapeutic agents used in the management of illnesses with a peripheral pain component with molecular masses similar to that of sucrose (342 Da), such as morphine (285 Da), codeine (300 Da), acetaminophen (150 Da), methotrexate (454 Da), fluoxetine (320 Da), amitripyline (278 Da), and cyclobenzaprine (276 Da), whose transport into the CNS may be different than seen in healthy individuals. (However, caution must be taken in extrapolating these findings to larger therapeutic agents, because the exact size of the BBB opening is not yet known.) Thus, this led us to the next study investigating codeine.

0 - 168 h BBB Permeability Time Course of [^{3}H] Codeine under λ-Carrageenan-Induced Inflammatory Pain

Now that we had demonstrated alterations in paracellular diffusion of a membrane impermeant marker, [^{14}C] sucrose over a time course, we next wanted to investigate how this would translate functionally to a clinically used drug under an inflammatory pain state.

Codeine is a moderate mu opioid receptor agonist and is prescribed for its antitussive and antinociceptive properties (Goodman et al., 2001). Its action is centrally-mediated thus any breakdown in BBB integrity can lead to significant consequences. Consequences that with high doses of most opioids in the CNS include respiratory depression, nausea, vomiting, dizziness, and mental clouding (Goodman et al., 2001). This is in addition to both addiction and acute tolerance, both of which can be profoundly influenced by higher than expected CNS doses. Thus, increased codeine uptake into the brain due to BBB disruptions may have serious implications.

To investigate the possibility of increased CNS dosing of codeine, we studied the BBB permeability of [^{3}H] codeine. Consistent with a previously cited codeine-to-morphine brain uptake ratio of 10-12:1 (Oldendorf et al., 1972), our control (0 h) R_{brain}% uptake value of codeine (39 ± 3.5%) was in the range of 5.7 ± 2.2%. The observed increase in codeine permeability to the CNS at 3 and 48 h (Figure 2.4) correlates well with [^{14}C] sucrose BBB permeability results (Figure 2.3). This is the first known observation that peripheral inflammation results in greater analgesic drug uptake to the brain. In addition, paracellular diffusion changes exhibited by [^{14}C] sucrose are consistent with those of [^{3}H] codeine based on the molecular masses of sucrose (342 Da) and codeine (300 Da), though molecular mass is not the only determinant of permeability (Egleton and Davis, 1997). BBB transport of codeine is a passive process, and since it is highly lipophilic, the penetration of codeine to the brain primarily depends on BBB transcellular permeability and blood flow (Bradbury et al., 1975; Xie and Hammarlund-Udenaes, 1998). Since capillary depletion studies demonstrated minimal vascular

volume changes, blood flow changes induced by λ-carrageenan inflammatory pain are unlikely, as previously reported (Huber et al., 2001).

The above studies demonstrate the significant effects of inflammatory pain states on paracellular diffusion of both a membrane impermeant marker and a common clinically used drug. Implications for neurotoxicity and improper dosing regimens of medications under inflammatory pathophysiological conditions point to the need for further study.

CHAPTER 3. THE EFFECT OF INFLAMMATORY PAIN ON ANALGESIA

Chapter studies published in:

Hau, V.S., Huber, J., Campos, C. and Davis, T.P.: Effect of λ-carrageenan induced inflammatory pain on codeine blood-brain barrier permeability and antinociception. Brain Research **1018(2)**: 257-64, 2004.

Introduction

Previous studies from have shown that peripheral inflammatory pain alters BBB function resulting in increased permeability of sucrose associated with an increase in paracellular diffusion (Chapter 2). Formalin-, λ-carrageenan-, and complete Freund's adjuvant-induced inflammatory pain models were shown to increase BBB permeability to sucrose (Figure 2.2). When λ-carrageenan-induced inflammatory pain was investigated over a 168 h time course, there was a biphasic increase in BBB permeability to [^{14}C] sucrose (membrane impermeant marker), at 1-6 h and 48 h (Figure 2.3). We have also shown that λ-carrageenan, when administered intravenously into the peripheral circulation, had no direct effect on BBB function (Chapter 3). These findings suggest λ-carrageenan-induced inflammatory pain causes alterations to BBB TJs. In this study, we investigated the effect of peripheral inflammatory pain on the BBB in terms of therapeutic drug delivery of codeine manifested by functional analgesia, as altered delivery of therapeutic agents may lead to ineffectual dosing and potential neurotoxicity.

Codeine was chosen for this examination because it is an analgesic drug commonly used for the treatment of inflammatory pain

Codeine, a moderate agonist for the mu opioid receptor, is prescribed for its antinociceptive and antitussive properties (Goodman et al., 2001). Therapeutic properties are centrally-mediated; thus, it is essential that codeine cross the BBB for it to reach its site of action (i.e. mu opioid receptor). Due to the high lipophilicity of codeine, BBB transport occurs via passive diffusion and is primarily dependent on BBB transcellular permeability and blood-flow (Bradbury et al., 1975; Xie and Hammarlund-Udenaes, 1998). As with most opioids, high doses of codeine in the CNS produce a wide spectrum of CNS-mediated side effects, including respiratory depression, nausea, vomiting, dizziness, and mental clouding (Goodman et al., 2001). It is well known that BBB integrity and P-gp expression of the opioid efflux pump affects sensitivity to opioids in terms of these side effects. For example, pre-delivery administration of a nontoxic level of morphine to an expecting mother, has resulted in the newborn infant exhibiting profound respiratory depression and analgesia because of immature BBB development (Way et al., 1965). In addition, opioids have been associated with acute tolerance and addiction, both of which can be exacerbated by BBB alterations resulting in higher than expected CNS doses. Thus, increased codeine uptake into the brain due to BBB disruptions may have serious implications. BBB perturbations may manifest clinically through functional changes in paracellular diffusion of codeine with increased analgesia and/or adverse side effects, thus, establishing the clinical significance of inflammatory pain-induced alterations of the BBB.

Methods

Chemicals

All chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO).

Animals and Treatments

Female Sprague-Dawley rats (250-300 g; Harlan Sprague Dawley, Indianapolis, IN) were housed under standard 12:12-h light-dark conditions and received food ad libitum. Protocols used in this study were approved by the University of Arizona Institutional Animal Care and Use Committee and abided by NIH guidelines. To induce inflammatory pain, rats were briefly anesthetized under 5% halothane gas, and either 3% λ-carrageenan or 0.9% saline (100 uL; s.c.) was injected into the plantar surface of the right hind paw.

Antinocieptive Analysis

For assessment of nociceptive profile, a radiant-heat tail flick analgesia meter (model 33; IITC Scientific Products; Woodland Hills, CA) (Figure 3.1) was used. Baseline latency was set at 2-3 s with a 15 s cut-off to avoid tissue damage. Fifteen minutes before the selected time points (both saline and λ-carrageenan treated) rats were administered 0.9% saline or codeine (dissolved in 0.9% saline) i.p. (7 mg/kg; 150 μl

bolus) at 3, 48, or 168 h post-λ-carrageenan or saline paw injection. Codeine dose and assessment time (15 min after saline or codeine i.p. injection) were based upon dose-time response studies conducted prior to this study in which a 40% maximal possible effect (% MPE) was observed using these parameters in control animals. Antinociception assessment was stopped at any time point within 10% of baseline.

Nociceptive sensitivity was measured by converting analgesic tail-flick times to % MPE:

% MPE = [(recorded flick time – baseline) ÷ (maximum time (15 s) – baseline)] X 100 %

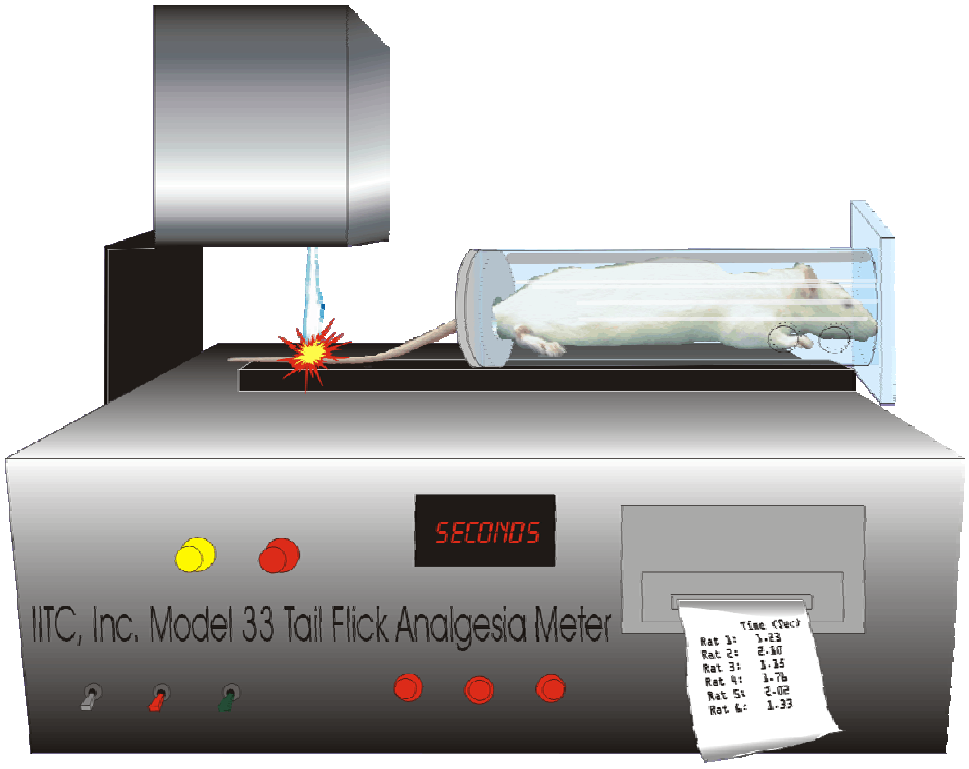

Figure 3.1 Rat Tail Flick Analgesia Meter

Data Analysis

In all experiments, data were presented as mean ± SE. Antinociception data were analyzed using two-way ANOVA to determine significance between time points, treatments, and interaction between (time x treatment). Pairwise comparisons for all significant groupings were performed using Tukey's HSD post hoc test. Significance was defined as $p<0.05$. All analyses were performed using the Sigma Stat 2.03 statistical software package (SPSS Inc.; San Rafael, CA).

Results

Antinociception Analysis

Figure 3.2 shows % MPE following codeine administration (7 mg/kg; i.p.) in rats with λ-carrageenan-induced inflammatory pain as compared to saline controls. Bars represent mean % MPE ± SE (n = 6). Antinociception was not significantly (p>0.05) different in codeine-treated naïve (no paw injection) as compared to codeine-treated saline control rats (data not shown). Also, no difference in tail flick antinociception was observed at any time point between non-codeine treated saline and non-codeine treated λ-carrageenan rats (p>0.05). Saline animals treated with codeine demonstrated a significant (p<0.01) increase in % MPE in all time points post injection as compared to non-codeine treated saline rats. λ-Carrageenan-animals treated with codeine demonstrated a significant (p<0.01) increase in % MPE at 3 and 48 h post injection as compared to codeine treated saline controls (44 ± 11% and 55 ± 9%, respectively). Difference between % MPE in codeine treated λ-carrageenan and codeine treated saline control rats at 168 h was not statistically (p>0.05) significant.

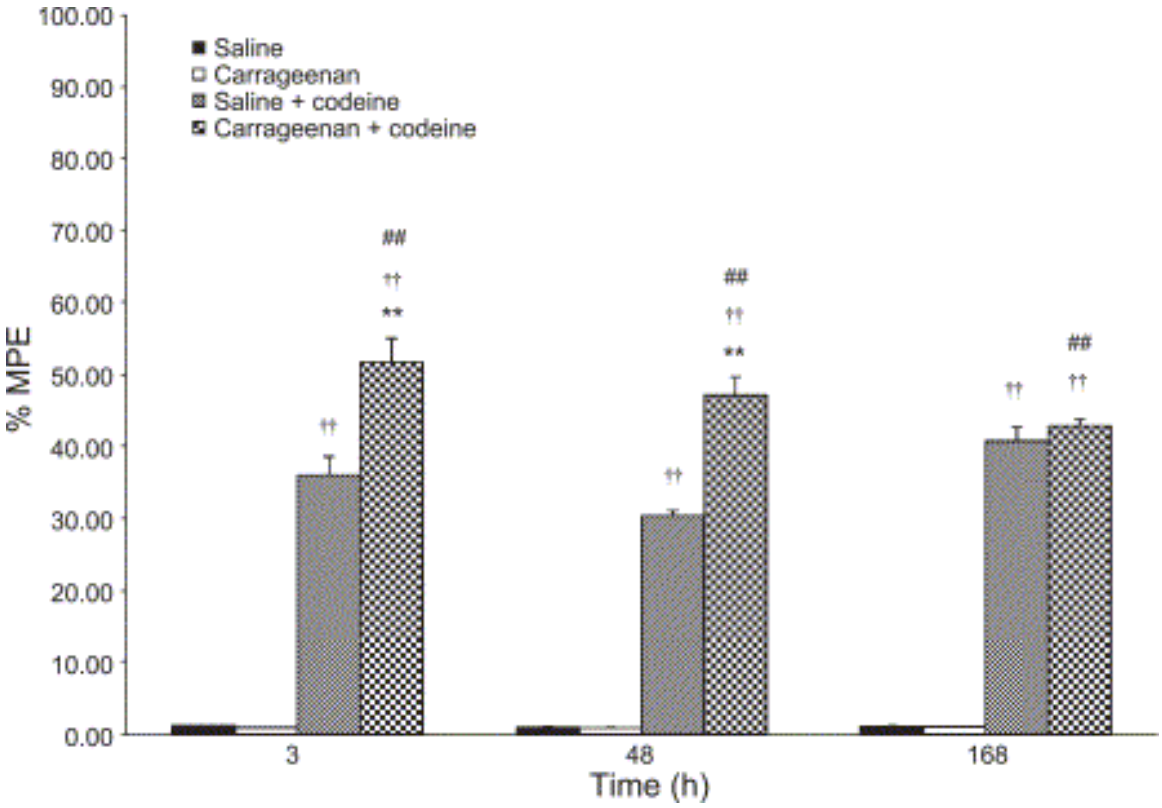

Figure 3.2 Codeine antinociception after λ-carrageenan-induced inflammatory pain. Bars represent mean %MPE±S.E. (*n*=6). Female Sprague–Dawley rats were administered an i.p. dose of 7 mg/kg of codeine. There was no difference in tail flick antinociception at any time point between non-codeine-treated saline and non-codeine-treated λ-carrageenan rats. Saline animals treated with codeine demonstrated a significant increase in %MPE in all time points post-injection as compared to non-codeine-treated saline rats. λ-Carrageenan animals treated with codeine demonstrated a significant increase in %MPE at 3 and 48 h post-injection as compared to codeine-treated saline controls, non-codeine-treated saline and non-codeine-treated λ-carrageenan rats. Significance was determined using a two-way ANOVA followed by Tukey's HSD post-hoc analysis. $^{**}p<0.01$ versus codeine-treated saline control; $^{\dagger\dagger}p<0.01$ versus non-codeine-treated saline; $^{\#\#}p<0.01$ versus non-codeine-treated λ-carrageenan.

Discussion

Currently, there is a growing body of research characterizing the BBB under normal physiological conditions; however, little is known about BBB regulation under pathophysiological conditions, such as inflammatory pain (Huber et al., 2001). In previous studies, we demonstrated the influence of peripheral inflammatory pain on blood-to-CNS uptake of the analgesic drug, codeine. We studied BBB permeability of both codeine and sucrose in λ-carrageenan-treated and saline control rats. With the increase in permeability that we observed our next question was what did this mean clinically. This has important consequences for dosing regimens of potentially neurotoxic or CNS-acting therapeutics for patients experiencing inflammatory pain.

In this study, we tested codeine antinociception in both groups of rats; saline control and λ-carrageenan-treated. Antinociception is a measurable functional endpoint demonstrating the outcome of increased codeine BBB permeability. In addition, it is relevant clinically as codeine is commonly used for inflammatory pain disorders.

We investigated the potential of codeine to elicit antinociception at the times of maximal increased [^{14}C] sucrose and [^{3}H] codeine permeability: 3 and 48 h (Figures 2.3 and 2.4). We chose a codeine dose (7 mg/kg) and assessment time (15 min) based upon 40% MPE in control rats to ensure our study observed both decreases and increases in antinociception. Nociceptive study results showed that rats treated with codeine experience enhanced antinociception under λ-carrageenan induced inflammatory pain as compared to saline controls, demonstrating a potential increase in CNS delivery of

codeine during peripheral inflammatory pain. The results showing increased [^{14}C] sucrose and [^{3}H] codeine permeability indicate a BBB compromise from λ-carrageenan induced inflammatory pain and when combined with enhanced codeine analgesia, suggests the possibility of a greater dose of codeine was delivered to the CNS. Although, further studies are needed to investigate this possibility and rule out other factors (i.e. endogenous opioid release by inflammatory mediators contributing to the enhanced analgesia).

Ion imbalances and therapeutic drug neurotoxicity can result from small paracellular changes in the BBB, such as those that would allow paracellular passage of sucrose and codeine. In fact, when lipopolysaccharide or TNF-α are given concurrently with cisplatin (a minimally BBB-penetrating chemotherapeutic agent) its levels are increased significantly in the cerebral cortex, likely via increased paracellular diffusion. This increase uptake of cisplatin is associated with neurotoxicity which has been attributed to a compromised BBB (Minami et al., 1998). Agents of similar molecular weight prescribed for the management of inflammatory pain-related illnesses, including morphine (258 Da), acetaminophen (150 Da), methotrexate (454 Da), and amitripyline (278 Da), can have significant CNS effects as well. We observed an increased CNS effect (analgesia) of codeine, and while we did not measure neurotoxicity, the potential exists since higher doses of opioids are associated with opioid neurotoxicity (Potter et al., 1989). Additionally, addiction and acute tolerance are characteristics of opioids which are known to be affected by dosing levels. Therefore incidence of addiction and acute

tolerance may be influenced by increased CNS levels of codeine or other opioids during inflammatory pain treatment.

This study demonstrates how pathophysiological conditions such as peripheral inflammation can have an effect on drug delivery across the BBB ultimately affecting its clinical purpose. It points to how consideration of the pathophysiological condition of the patient may be important to take into account before prescribing highly neurotoxic medications.

CHAPTER 4: EFFECT OF INFLAMMATORY AGENTS DIRECTLY ON THE BLOOD-BRAIN BARRIER

Chapter studies published in:

Huber, J.D., **Hau, V.S.**, Mark, K.S., Brown, R.C., Campos, C.R. and Davis, T.P.: Viability of *in vitro* and *in vivo* microvascular endothelial cells to direct exposure of formalin, λ-carrageenan, and complete Freund's adjuvant. *Eur J Pharmacol*. **450 (3)**: 297-304, 2002.

Introduction

Research involving peripheral inflammation has been used for several decades to assess pain neurobiology and its contributions have created a number of well-characterized pain models to evaluate the profile of nociceptive, behavioral, and physiological components of noxious stimuli (Winter et al., 1962; Brown et al., 2001; Porro and Cavazzuti, 1993; Lidow et al., 2001).

To date, most research using pain models has focused on specific parameters of pain (such as nociception, immune response, neurotransmitter release, etc.) or the efficacy of anti-inflammatory drugs (Saleh et al., 1996; Gilbert and Franklin, 2001; Lidow et al., 2001). Recently, we evaluated three well-characterized inflammatory pain models (formalin, λ-carrageenan, and complete Freund's adjuvant) to determine their effects on cerebral microvascular beds. These inflammatory pain models have different onsets of action, duration times, and physiological responses at the site of action. Our

current findings show that all three inflammatory agents affected blood–brain barrier permeability (Chapter 2). The structural and functional changes seen during systemic inflammatory pain have thus far been credited to the pathophysiological response of the inflammatory agents through a combination of immune, hormonal, and neuronal responses. However, a primary concern exists as to whether the inflammatory agents can elicit a direct effect on the blood–brain barrier by absorbing into the systemic circulation from the site of injury and subsequently affect cerebral microvascular endothelial cells.

Using primary cultured bovine brain microvessel endothelial cells, we assessed the cellular viability and paracellular permeability of a confluent monolayer in the presence of each of the three inflammatory agents. The high concentration of each agent was calculated as the amount present if the entire bolus given via a subcutaneous injection into the paw were absorbed into the peripheral circulation at once. Lower concentrations of each substance were assessed to determine if any effects would be seen at levels several fold less than maximal, which would be more indicative of absorption from the site of injection. Furthermore, we assessed the functional and structural integrity of an intact blood–brain barrier using an *in situ* brain perfusion and Western blot analyses of isolated cerebral microvessels following an i.v. bolus of an inflammatory agent at a concentration equivalent to the amount injected subcutaneously into the hind paw. The results of this investigation clearly show that the functional and structural alterations seen at the blood–brain barrier following a peripheral inflammatory insult are due to physiological actions in response to the inflammatory pain and not due to any direct

interaction between the blood–brain barrier microvasculature and the inflammatory-inducing agent.

Methods

Radioisotopes/Antibodies/Materials

[^{14}C] Sucrose was obtained from ICN Pharmaceuticals (specific activity: 492 mCi/mmol, >99.5% purity; Irvine, CA). Primary antibodies (anti-zonula occludens-1, anti-occludin, anti-claudin 1) were obtained from Zymed (San Francisco, CA). Conjugated anti-rabbit immunoglobulin G and anti-mouse immunoglobulin G-horseradish peroxidase were purchased from Amersham Life Science Products (Springfield, IL). Anti-actin and all other chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO).

Animals and Treatments

Female Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 250–300 g were housed under standard 12 h light/dark conditions and received food ad libitum. All protocols involving animals were approved by the University of Arizona Institutional Animal Care and Use Committee and abide by NIH guidelines. Rats were anesthetized with sodium pentobarbital (60 mg/kg; i.p.) and subsequently injected (100 μl; i.v.) with inflammatory agent (5% formalin, 3% λ-carrageenan, or 50% complete Freund's adjuvant) into the tail vein and placed into two groups. Concentrations of inflammatory agents were equivalent to the amount commonly injected into the hind paw when conducting nociceptive testing. The first group of animals underwent a 20-min *in situ* brain perfusion at 1, 3, or 72 h post-injection, respectively. These time points represent

commonly used time points for nociceptive testing of each agent (Winter et al., 1962; Nozaki-Taguchi and Yamamoto, 1998; Wei et al., 1999). Animals from the second group were anesthetized with sodium pentobarbital and the brains were harvested at 1, 3, or 72 h for Western blot analyses. Control animals were injected (100 μl, i.v.) with 0.9% saline into the tail vein. Naïve controls showed no significant difference in BBB alterations compared to the saline-treated controls and were therefore not included in this study.

Bovine Brain Microvessel Endothelial Cell Isolation

Fresh bovine brains were obtained from the University of Arizona Animal Sciences Meat Laboratory. Bovine brain microvessel endothelial cells were isolated from cerebral cortex gray matter and cryopreserved, as previously described (Audus and Borchardt, 1987). Isolated cells were seeded at a cell density of 50,000 cells/cm onto rat tail collagen and fibronectin coated Transwells® containing a 25 mm polyester membrane insert with a 0.4 μm pore size or 12-well tissue culture plates (Costar Nucleopore, Cambridge, MA). Bovine brain microvessel endothelial cells were grown to confluent monolayers (10–12 days) prior to being used for cell viability or transendothelial cell permeability studies. Endothelial cells used for these studies were primary cultured cells, which have been shown to maintain excellent blood–brain barrier characteristics as well as a good *in vivo* correlation (i.e., 0.75) (Weber et al., 1993; Brownson et al., 1994; Abbruscato et al., 1997; Abbruscato and Davis, 1999).

Endothelial Cell Viability Assay

Viability of bovine brain microvessel endothelial cells following exposure to formalin, λ-carrageenan, or complete Freund's adjuvant was assessed using the MTT (3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide) cytotoxicity assay (Hansen et al., 1989). Confluent bovine brain microvessel endothelial cell monolayers were exposed to formalin (0.025% and 0.0025%), λ-carrageenan (0.015% and 0.0015%), or complete Freund's adjuvant (0.25% and 0.025%) for (1, 3, and 72 h, respectively). Cell culture medium was removed and cells were rinsed with phosphate buffered saline (PBS) and incubated with 200 µl of MTT (5 mg/ml) for 2 h at 37 °C. Excess MTT was removed and cells rinsed with PBS prior to solubilizing with 200 µl of a 50:50 mixture of dimethyl formamide and 20% (w/v) sodium lauryl sulfate (pH 4.7). Absorbance readings were taken at 550 nm using a Lab systems Multiskan RC™ microplate reader (Fisher, Tustin, CA). Cell viability was expressed as a percent of control bovine brain microvessel endothelial cells (untreated).

Bovine Brain Microvessel Endothelial Cell Monolayer Permeability to [^{14}C] sucrose

Passage of [^{14}C] sucrose across bovine brain microvessel endothelial cell monolayers were used to assess paracellular permeability changes induced by three inflammatory pain models, formalin (0.025%, 0.0025%, 0.00025%), λ-carrageenan (0.015%, 0.0015%, 0.00015%), or complete Freund's adjuvant (0.25%, 0.025%, 0.0025%) for 1, 3, or 72 h, respectively. Following treatment, confluent bovine brain microvessel endothelial cell monolayers were incubated with assay buffer, consisting of (122 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM

HEPES, 10 mM glucose and 0.4 mM K_2HPO_4) for 30 min at 37 °C. Paracellular permeability across bovine brain microvessel endothelial cell monolayers was determined by adding [^{14}C] sucrose (0.5 μCi) to the luminal side (upper compartment of Transwell®) and samples (50 μl) were removed from the abluminal side (lower chamber of Transwell®) at 0 and 120 min and replaced with fresh assay buffer. Concentrations of [^{14}C] sucrose applied to the luminal side were determined by removing samples (50 μl) at time zero. Amount of radioactivity in the samples from permeability studies was determined using a Beckman LS5000 TD beta-counter (Fullerton, CA). Permeability coefficients (PC) for [^{14}C] sucrose were expressed as previously described (Deli et al., 1995) where V=volume in receiver chamber (1.5 cm^3), SA=surface area of cell monolayer (1 cm^2), C_d=concentration of marker in donor chamber at time zero, and C_r=concentration of marker in receiver at time (T).

$$\mathbf{PC\ (cm/min) = [(V / (SA \times C_d)) \times (C_i / T)]}$$

In situ Brain Perfusion

Following treatment with inflammatory agents, rats were anesthetized, as above, and heparinized (10,000 U/kg). Body temperature was maintained using a heating pad. The right common carotid artery was exposed and cannulated with silicone tubing connected to a perfusion circuit. Perfusate consisted of a modified mammalian Ringer's solution (117 mM NaCl, 4.7 mM KCl, 0.8 mM $MgSO_4$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 10 mM D-glucose, dextran [MW 70,000; 10 g/l]; bovine serum

albumin-type V, 1 g/l, pH 7.4) (Preston et al., 1995). Addition of Evan's blue (55 mg/l) to the Ringer's solution provided a control for blood–brain barrier integrity. Perfusate was aerated with 95% O_2/5% CO_2, and warmed to 37 °C. The ipsilateral vein was sectioned to allow drainage. Once the desired perfusion pressure and flow-rate were achieved (85–95 mm Hg; 3.1 ml/min, respectively), the contralateral carotid artery was cannulated and perfused as described above. Radiolabeled sucrose was infused into the inflow of the perfusate using a slow-drive syringe pump (0.5 ml/min/hemisphere; model 22: Harvard Apparatus, South Natick, MA) for 20 min (Figure 2.1). The animal was decapitated and brain was removed. Choroid plexi and meninges were excised and cerebral hemispheres sectioned and homogenized. Perfusate containing the radiolabeled marker was collected from each carotid cannula at the termination of the perfusion to serve as a reference.

Cerebral hemispheres (~500 mg) and 100 μl of perfusate were prepared for liquid scintillation counting by adding 1 ml of tissue solubilizer (TS-2; Research Products International, Mount Pleasant, IL). After 2 days of solubilization, 100 μl of 30% glacial acetic acid was added to eliminate chemiluminescence. Four milliliters of Budget Solve Liquid Scintillation Cocktail (Research Products International) were added and samples measured for radioactive counts (model LS 5000 TD Counter; Beckman Instruments).

Capillary Depletion

Measurement of the vascular component to total brain uptake was performed using capillary depletion (Triguero et al., 1990). After a 20 min *in situ* brain perfusion, the brain was removed and the choroid plexi and meninges excised. Homogenization

procedures were performed within 2 min of sacrificing the animal. Brain tissue (50 mg, wet weight) was homogenized (Polytron homogenizer; Brinkman Instruments, Westbury, NY) in 1.5 ml of capillary depletion buffer [10 mM 4-(2-hydroxyethyl)-piperaxineethane sulfonic acid; 141 mM NaCl; 4 mM KCl; 2.8 mM $CaCl_2$; 1 mM $MgSO_4$; 1 mM NaH_2PO_4; 10 mM, D-glucose; pH 7.4] and kept on ice. Two milliliters of ice-cold 26% clinical grade dextran was added and homogenization repeated. Aliquots of homogenate were centrifuged at 5400×*g* for 15 min. Capillary-depleted supernatant was separated from the vascular pellet. Homogenate, supernatant, and pellet were taken for radioactive counting. The amount of [^{14}C] sucrose in the brain homogenate, supernatant, and pellet was expressed as the percentage ratio of tissue (C_{Tissue} disintegrations/min/g of disintegrations/min/ml) to perfusate activities ($C_{\text{Perfusate}}$ disintegrations/min/ml) and expressed as R_{Brain}%.

$$\mathbf{R_{brain}\% = (C_{brain}/\ C_{perfusate})\ x\ 100\%}$$

Microvessel Isolation

At each time point, following inflammatory insult, the rats were anesthetized with sodium pentobarbital (60 mg/kg), decapitated, and their brains removed. Meninges and choroid plexi were excised and the cerebral hemispheres were homogenized in 4 ml of microvessel isolation buffer (103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES, 2.5 mM $NaHCO_3$, 10 mM D-glucose, 1 mM sodium pyruvate, dextran [MW 64,000; 10 g/l]; pH 7.4) with a protease inhibitor cocktail

(0.2 mM phenylmethylsulfonyl fluoride; 1 μM benzamide; 1 mM $NaVO_4$; 10 mM NaF; 10 mM sodium pyrophosphate; and 10 mg/ml of aprotinin and leupeptin). Four milliliters of 26% dextran were added and the homogenates vortexed. Homogenates were centrifuged at 5600×*g* for 10 min and the supernatant aspirated. Pellets were resuspended in 10 ml of microvessel isolation buffer and passed through a 100-μm filter (Falcon, Becton–Dickinson; Franklin, NJ). The filtered homogenates were centrifuged at 3000×*g*. Protein was extracted from the pellets using 6 M urea lysis buffer (6 M urea; 0.1% Triton X−100; 10 mM Tris, pH 8.0; 1 mM dithiothreitol; 5 mM $MgCl_2$; 5 mM EGTA; 150 mM NaCl) with the protease inhibitor cocktail. Protein concentrations were determined by bicinchoninic acid protein assay (Pierce, Rockford, IL).

Tight Junctional Protein Analysis

Isolated microvessel homogenates were analyzed for expression of occludin, zonula occludens-1, claudin-1, and actin using Western blot. Microvessel samples (20 μg) were resolved on a 4–12% Tris–glycine gel (Novex, San Diego, CA) for 90 min at 125 V and transferred to a polyvinylidene difluoride membrane for 40 min at 240 mA. Gel-Stain Blue (Pierce) was used to insure proper protein loading. Polyvinylidene difluoride membranes were blocked in Tris-buffered saline (141 mM NaCl, 10 mM Tris-base, 0.1% Tween-20) with 5% non-fat milk for 4 h. Blots were incubated in primary antibody at room temperature for 2 h, rinsed in Tris-buffered saline with 5% non-fat milk for 1 h, and incubated with secondary antibody at room temperature for 30 min. Blots

were developed using enhanced chemiluminescence (ECL+; Amersham Life Science Products) and analyzed using Scion Image (Scion, Frederick, MD).

Results

MTT Cell Viability

Bovine brain microvessel endothelial cells were exposed to formalin (1 h), λ-carrageenan (3 h), or complete Freund's adjuvant (72 h) and cell viability was assessed using an MTT assay (Figure 4.1). Cell viability was significantly ($P<0.01$) decreased at the high concentration of formalin (0.025%) resulting in a 34% decrease in cell viability. No significant difference occurred between any other treatment group when compared to the control group ($P\geq0.05$).

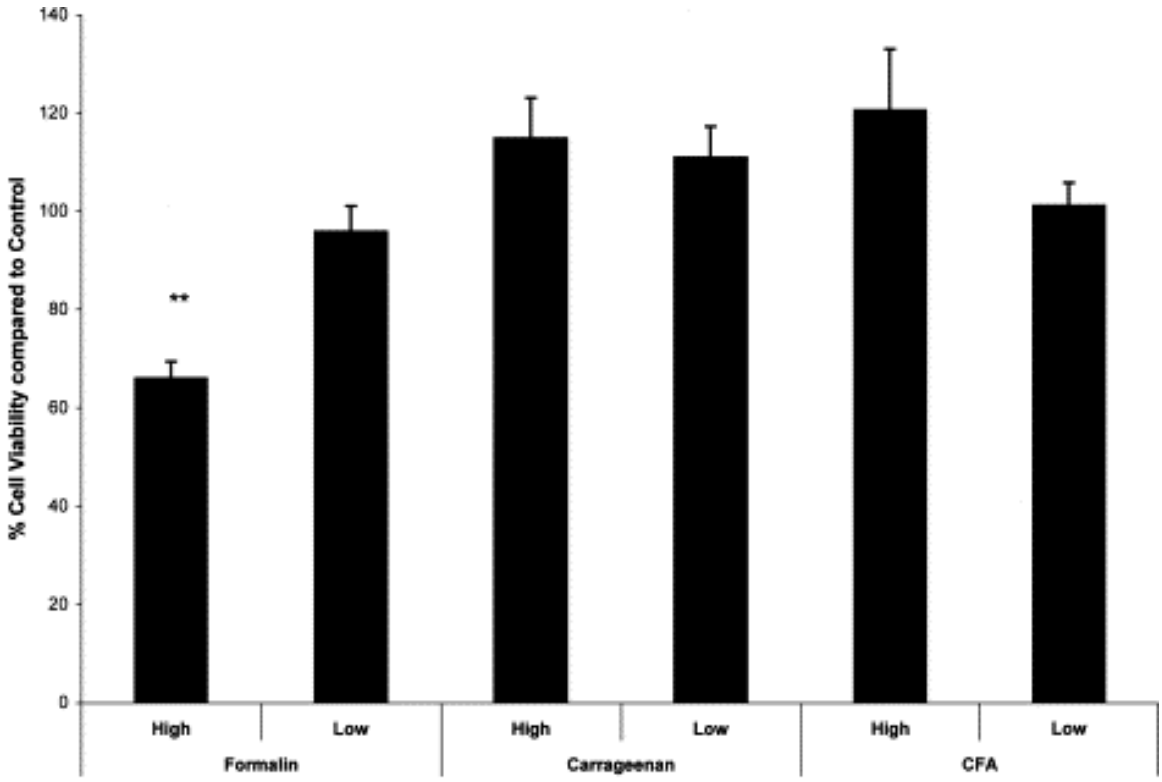

Figure 4.1 MTT cell viability study conducted on bovine brain microvessel endothelial cell monolayers (primary culture, 10–12 days confluent) following incubation in media conditioned with an inflammatory agent (formalin, λ-carrageenan, or complete Freund's adjuvant). High (0.025%, 0.015%, and 0.25%, respectively) and low (0.0025%, 0.0015%, and 0.025%, respectively) concentrations of each inflammatory agent were added to the media for a preset period of time (formalin–1 h, λ-carrageenan–3 h, and complete Freund's adjuvant—72 h). The high concentration of each inflammatory agent was designated as the amount present if the entire amount given via a subcutaneous injection into the paw were absorbed into the peripheral circulation at once, while the lower concentration was used to determine if any effects may be seen at levels several fold less than maximal. Each bar represents mean±S.E.M. (n=6). Statistical significance was determined using two-way ANOVA followed by Tukey's HSD post hoc test. ** Indicates (P<0.01) significance between treatments as compared to control. No significant interactions were observed.

Bovine Brain Microvessel Endothelial Cell Monolayer Permeability to [^{14}C] sucrose

Effects of exposure to inflammatory agents (formalin, λ-carrageenan, or complete Freund's adjuvant) for a preset incubation period (1, 3, and 72 h, respectively) were studied using bovine brain microvessel endothelial cell permeability to [^{14}C] sucrose. Figure 4.2 shows the calculated permeability coefficients for each agent at various doses. Results indicate the high concentration (0.025%) of formalin significantly (P<0.01) increased bovine brain microvessel endothelial cell monolayer permeability to [^{14}C] sucrose at 120 min with a permeability coefficient of 4.22±0.18 as compared to 3.08±0.07 for the control.

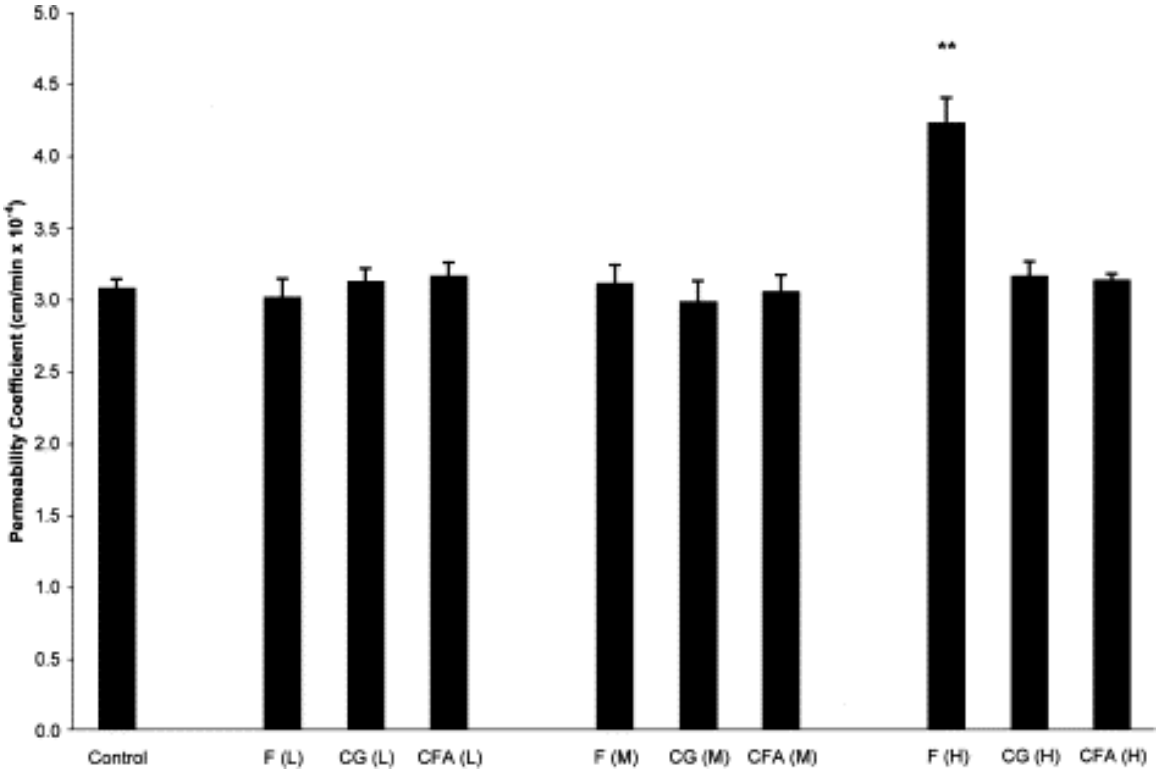

Figure 4.2 *In vitro* assessment of permeability across bovine brain microvessel endothelial cell monolayers (primary culture, 10–12 days) using [^{14}C] sucrose following incubation in media conditioned with an inflammatory agent (formalin, λ-carrageenan [CG], or complete Freund's adjuvant [CFA]) at 120 min. Low (L) (0.00025%, 0.00015%, and 0.0025%, respectively), medium (M) (0.0025%, 0.0015%, and 0.025%, respectively) and high (H) (0.025%, 0.015%, and 0.25%, respectively) concentrations of each inflammatory agent were added to the media for a preset period of time (formalin—1 h, λ-carrageenan—3 h, and complete Freund's adjuvant—72 h). The highest concentration of each inflammatory agent was designated as the amount present if the entire amount given via a subcutaneous injection into the paw were absorbed into the peripheral circulation at once, while the lower concentrations were used to assess bovine brain microvessel endothelial cell permeability at levels several fold less than maximal. Each bar represents mean±S.E.M. (n=4). Statistical significance was determined using two-way ANOVA followed by Tukey's HSD post hoc test. No significant interactions were observed. ** Indicates (P<0.01) significance between treatments as compared to control.

In situ Brain Perfusion

Effects of inflammatory agent (formalin, λ-carrageenan, or complete Freund's adjuvant) exposure for a preset incubation period (1, 3, or 72 h, respectively) on basal permeability across an *in vivo* blood–brain barrier were assessed using *in situ* perfusion of the brain with [^{14}C] sucrose, a membrane impermeant marker. Visual inspection of the brain immediately following *in situ* perfusion showed no influx of Evan's blue albumin into the brain parenchyma.

Figure 4.3 shows R_{Br}% for the treated and control groups. Control R_{Br}% value of 2.12±0.23, representative of vascular space volume, was converted to a vascular space of 21.2 μl/g brain tissue. Results demonstrate no significant difference in amount of sucrose associated with the brain (formalin—92.6±10.9, λ-carrageenan—92.2±10.2, complete Freund's adjuvant—86.2±6.8% compared to control, respectively). Capillary depletion data following the 20 min *in situ* brain perfusions showed the amount of [^{14}C] sucrose trapped in the pellet was not significantly different from control (Table 4.1). Furthermore, the study revealed the percentage amount of [^{14}C] sucrose associated with actual entry into the brain parenchyma was not statistically different from the homogenate (Table 4.1).

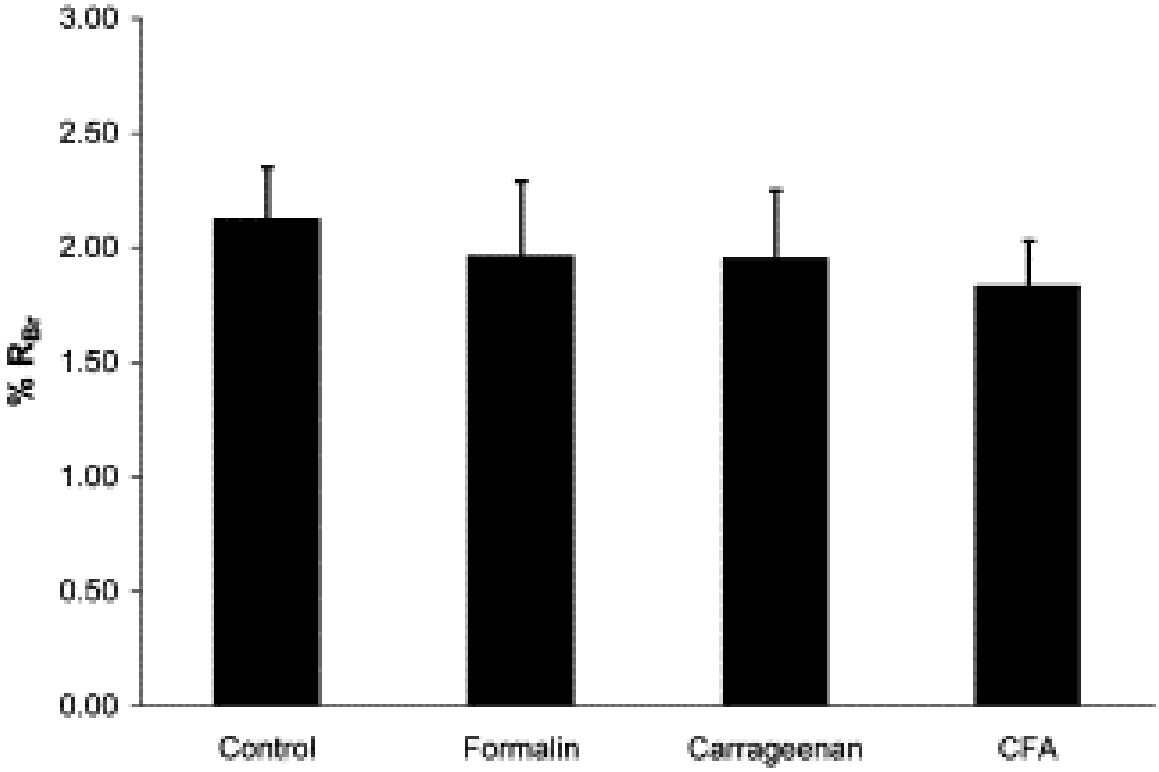

Figure 4.3 [^{14}C] Sucrose blood–brain barrier permeability after i.v. injection of inflammatory agent (formalin, λ-carrageenan, or complete Freund's adjuvant) into the tail vein. Concentrations of inflammatory agents are equivalent to the amount commonly injected into the hind paw when conducting nociceptive testing. After a preset period of time (formalin—1 h, λ-carrageenan—3 h, and complete Freund's adjuvant—72 h), a 20 min *in situ* brain perfusion was performed. R_{Br}%, ratio of radioactivity found in the brain compared with the radioactivity found in the perfusate media. Each bar represents mean±S.E.M. (n=4).

Table 4.1 Capillary depletion studies after a 20 min *in situ* brain perfusion

Model	Pellet	Supernatant	Homogenate
Control	$0.34 \pm 0.12^{\dagger}$	1.84 ± 0.41	1.87 ± 0.53
Formalin	$0.25 \pm 0.16^{\dagger}$	1.61 ± 0.16	1.57 ± 0.30
λ-Carrageenan	$0.31 \pm 0.09^{\dagger}$	1.45 ± 0.06	1.50 ± 0.17
CFA	$0.11 \pm 0.04^{\dagger}$	1.36 ± 0.16	1.40 ± 0.14

Values are mean ± S.E.M.; n=3. Data are the percent values after a 20 min *in situ* brain perfusion with [^{14}C] sucrose. Statistical significance was determined using two-way ANOVA. No significant difference was determined between treatment groups and no significant interactions were observed ($P \geq 0.05$). $^{\dagger}P < 0.01$, significant difference from homogenate.

Tight Junctional Protein Analysis

Western blot analyses indicated no significant change in expression of the tight junctional proteins (occludin, zonula occludens-1, and claudin-1) and the cytoskeletal protein (actin) following i.v. injection of inflammatory agents (formalin, λ-carrageenan, and complete Freund's adjuvant) for a preset period (1, 3, and 72 h, respectively). Figure 4.4 A-D shows the relative difference (%) from control of optical density for each protein.

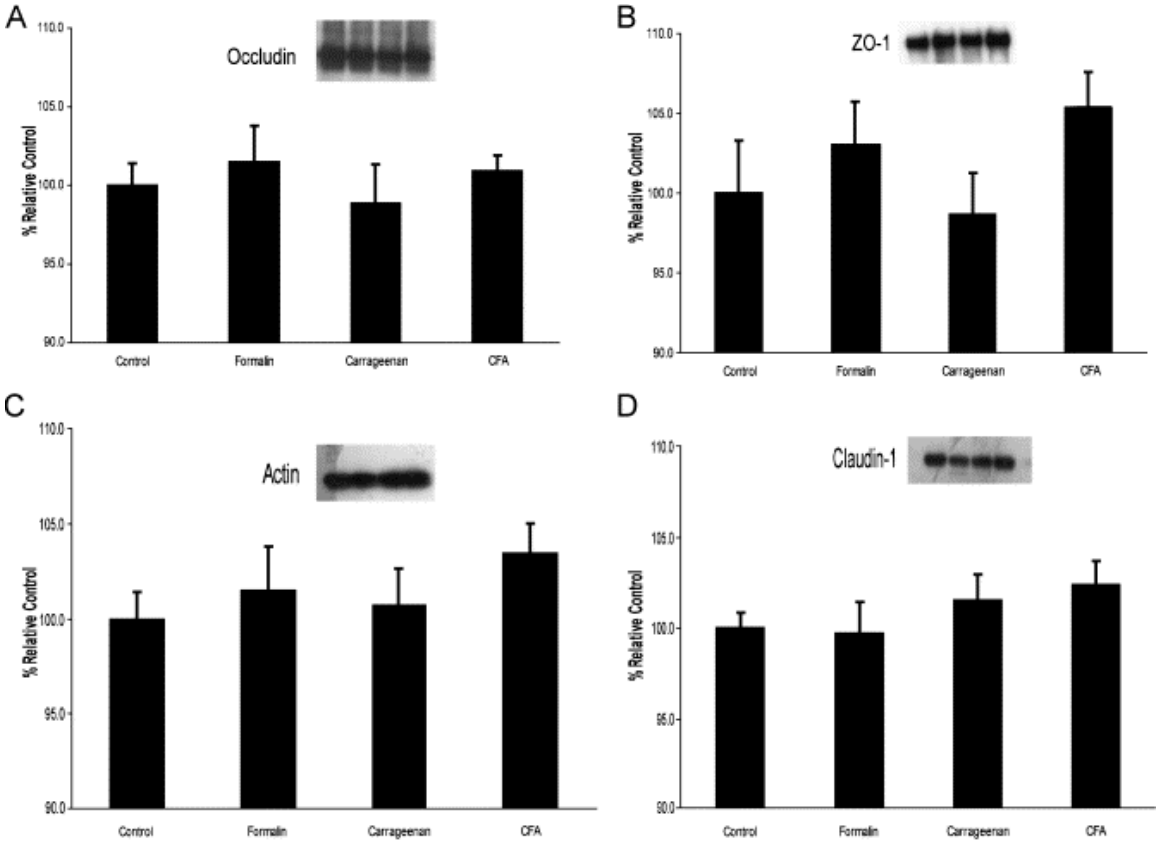

Figure 4.4 Western blot analyses of isolated microvessels indicate that expressions of tight junctional proteins are not altered by i.v. injection of an inflammatory agent, (formalin, λ-carrageenan, or complete Freund's adjuvant) into the tail vein. (A) occludin, (68 kDa); (B) zonula occludens-1, (220 kDa); (C) actin, (42 kDa) (D) claudin-1, (46 kDa). Insets: representative Western blots. Lane 1—control, lane 2—formalin, lane 3—λ-carrageenan, lane 4—complete Freund's adjuvant. Each bar represents mean±S.E.M. (*n*=3).

Discussion

In this study, we investigated three inflammatory agents to establish if these agents elicited a direct effect on the functional and structural integrity of the blood–brain barrier. Using *in vitro* and *in situ* techniques, we were able to evaluate the effect of these inflammatory agents on cell viability, permeability, and tight junctional protein expression. In previous studies, we have shown that formalin, λ-carrageenan, and complete Freund's adjuvant, when administered subcutaneously into the hind paw, induced a time-dependent increase in blood–brain barrier permeability (Figure 2.2). Furthermore, we showed that hind paw injection of λ-carrageenan produced a biphasic increase in blood–brain barrier permeability, occurring from 1 to 6 h and then again at 48 h (Figure 2.3). However, to confirm that the observed changes were elicited by the inflammatory pain and not directly due to the inflammatory agents entering the systemic circulation and affecting the cerebral microvasculature of the blood–brain barrier, these experiments were conducted.

MTT cytotoxicity assays were performed to determine the viability of bovine brain microvessel endothelial cells exposed to the inflammatory agents at concentrations equivalent to that potentially in the systemic circulation following injection into the hind paw, if it were to entirely enter at the same time. Results indicate that only the highest concentration of formalin (0.025%) affected bovine brain microvessel endothelial cell viability (~34% cytotoxic) (Figure 4.1). Subsequently, we investigated the effect of these same agents on bovine brain microvessel endothelial cell permeability. The results again

showed that only the highest concentration of formalin affected the permeability coefficient of bovine brain microvessel endothelial cell monolayers (Figure 4.2), which is directly related to the decreased cell viability seen at this same concentration. Therefore, bovine brain microvessel endothelial cells appear to be viable and functional in media containing the inflammatory agents at concentrations much higher than would be found in the systemic circulation.

We next assessed the basal permeability across an intact blood–brain barrier following an i.v. injection of formalin, λ-carrageenan, or complete Freund's adjuvant into the tail vein. I.v. route of administration was used to investigate the maximum potential exposure (although highly unlikely) of the rat BBB to an inflammatory agent if leakage into the peripheral circulatory system occurred from the injected paw. After a preset period of time (formalin—1 h, λ-carrageenan—3 h, and complete Freund's adjuvant—72 h), a 20 min *in situ* brain perfusion was performed. The control R_{Br}% value of 2.12±0.23 was converted to a vascular space of 21.2 μl/g brain tissue, which is similar to our previous studies and to other studies using vascular space markers (Blasberg et al., 1983; Williams et al., 1996). No significant difference was noted in the amount of sucrose associated with the brain in any of the treated groups compared to control, indicating no change in blood–brain barrier permeability after a direct i.v. bolus.

Finally, we investigated the structural integrity of the tight junctional complex between blood–brain barrier endothelial cells using Western blot analyses. Tight junctions form a rate-limiting barrier between the extracellular fluid surrounding brain parenchyma and the systemic circulation. Tight junctions are primarily comprised of two

transmembrane proteins, claudins and occludin, which attach one endothelial cell to another, and several accessory proteins, including zonula occludens-1, which form a scaffold to attach the tight junction complex to the cytoskeleton (actin). As has previously been discussed (Chapter 1) changes in these proteins indicate a significant alteration in blood–brain barrier structural integrity that can be correlated with functional changes. In this study, we have focused our attention on these four proteins: claudin, occludin, zonula occludens-1, and actin. In these studies, Western blot analyses show no significant difference in expression of any of the chosen proteins following i.v. injection of the inflammatory agent, indicating that the effects of inflammatory pain on the blood–brain barrier are most likely mediated by the inflammatory response and its effector molecules rather than the inflammatory agent itself.

In summary, we have clearly shown that the effects on blood–brain barrier functional and structural integrity seen in our previous studies are not due to a direct chemical interaction between the inflammatory agent and the cerebral endothelial cells of the BBB. Furthermore, this study shows that, at the concentrations used to elicit peripheral inflammation (either i.p. or s.c.), none of the inflammatory agents investigated are systemically toxic, which was a great concern especially for the formalin model (Pandey et al., 2000). This study helps to confirm that the changes seen at the blood–brain barrier following inflammation are due to the physiological response to the given inflammatory agent. By using three different inflammatory pain models, we are able to explore many different facets of the pain response (onset, time course, neuronal/immune contributions, etc.) and insure that any novel findings are not solely an artifact of a single

pain model. Finally, this study strengthens these inflammatory pain models as experimental tools for blood–brain barrier assessment during pain.

CHAPTER 5. EFFECT OF INFLAMMATORY PAIN ON BLOOD-BRAIN BARRIER TIGHT JUNCTION PROTEIN EXPRESSION

Chapter studies published in:

Hau, V.S., Huber, J., Campos, C. and Davis, T.P.: Effect of λ-carrageenan induced inflammatory pain on codeine blood-brain barrier permeability and antinociception. Brain Research **1018(2)**: 257-64, 2004.

Huber, J.D., **Hau, V.S.**, Campos, C.R., Egleton, R.D., and Davis, T.P.: Functional and structural analysis of blood-brain barrier tight junctions during a 72 hr exposure to λ-carrageenan induced inflammatory pain. Am J Physiol Heart Circ Physiol **283 (4)**: H1531-7, 2002.

Introduction

Currently there are many studies investigating the molecular structure of tight junctions. However, studies that focus on understanding the regulation of tight junctions under physiological and pathophysiological conditions are much less.

As discussed previously (Chapter 1), tight junction strands are primarily composed of two distinct four transmembrane proteins, claudin and occludin. Claudins form dimers that bind homotypically to adjacent endothelial cells to form the primary seal of the tight junction (Furuse et al., 1998). Claudins comprise a multigene family, and, to date, there are 20 claudin subtypes identified (Tsukita and Furuse, 2000). Occludin, once believed to be the major tight junction protein, is found in high concentrations at BBB tight junctions (Hirase et al., 1997); however, occludin is not necessary for tight junction

formation (Morita et al., 1999). Rather, a previous study (Balda et al., 2000) has shown occludin presence increases electrical resistance across the junction.

Tight junctions also consist of several accessory proteins necessary to form structural support. The zonula occluden (ZO) proteins (ZO-1, ZO-2, and ZO-3) belong to the membrane-associated guanylate kinase-like proteins (MAGUKs), a family of proteins (Haskins et al., 1998; Itoh et al., 1999) that serve as recognition proteins for tight junctional placement and support structures for signal transduction proteins (Haskins et al., 1998). Figure 1.2 illustrates the proposed interactions of the major proteins associated with tight junctions at the BBB.

To date, little is known about peripheral inflammation influences on the BBB. In an effort to further understand the cellular mechanisms associated with inflammatory pain, we used three well-characterized and established inflammatory pain models in the rat. Inflammation was produced by unilateral subcutaneous injection of formalin, λ-carrageenan, or complete Freund's adjuvant (CFA) into the right hind paw. Each of these models is characterized by a different onset and time course of inflammatory response. With the use of this approach, we compared each pain model to determine whether peripheral inflammation has an effect on expression of tight junctional proteins. We then further investigated the effects of λ-carrageenan-induced inflammatory pain on the functional and structural integrity of BBB tight junctions over a time course from 0 to 72 h and evaluated the correlation between increased BBB permeability and alterations in occludin and ZO-1 protein expression.

Methods

Antibodies and Chemicals

Primary antibodies (anti-ZO-1, anti-ZO-2, and anti-occludin, anti-actin, and anti-claudin) were obtained from Zymed (San Francisco, CA). Conjugated anti-rabbit IgG- and anti-mouse IgG-horseradish peroxidase were purchased from Amersham Life Science Products (Springfield, IL). Anti-actin and all other chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO).

Animals and Treatments

Female Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 250-300 g were housed under standard 12:12-h light-dark conditions and received food ad libitum. All protocols used in this study were approved by the University of Arizona Institutional Animal Care and Use Committee and abide by NIH guidelines. Rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and subsequently injected (100 μl sc) with selected inflammatory agent into the plantar surface of the right hind paw. Pentobarbital sodium was used in this study to insure no interference with *N*-methyl-D-aspartate receptor activity. At 1-h postinjection, the 0.9% saline control and 5% formalin-injected rats had their brains harvested for Western blot analyses. λ-Carrageenan (3%)- and CFA (50%)-injected rats underwent the same procedures at 3 h and 3 days, respectively. For the time course study, after rats were anaesthetized as above they were subsequently injected (100 μl sc) with 3% λ-

carrageenan into the plantar surface of the right hind paw. At 1, 3, 6, 12, 24, 48, or 72 h postinjection brains were harvested, and protein isolated for Western blot analyses. Control animals were injected (100 μl sc) with 0.9% saline into the plantar surface of the right hind paw. Naïve controls showed no significant difference in BBB alterations compared with the saline-treated controls and are therefore not included in this study.

Microvessel Isolation

At each time point after inflammatory insult, rats were anesthetized with pentobarbital sodium and decapitated, and the brains were removed. The meninges and choroid plexi were excised, and the cerebral hemispheres were homogenized in 4 ml microvessel isolation buffer [containing 103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES, 2.5 mM $NaHCO_3$, 10 mM D-glucose, 1 mM sodium pyruvate, and 10 g/l dextran (mol wt 64,000); pH 7.4] with protease inhibitor cocktail (0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 1 mM $NaVO_4$, 10 mM NaF, 10 mM sodium pyrophosphate, and 10 μg/ml aprotinin and leupeptin). Four milliliters of ice-cold 26% dextran were added, and the homogenates were vortexed. Homogenates were centrifuged at 5,600 *g* for 10 min, and the supernatant was aspirated. Pellets were resuspended in 10-ml microvessel isolation buffer and passed through a 100-μm filter (Falcon, Becton-Dickinson; Franklin, NJ). The filtered homogenates were centrifuged at 3,000 *g*. Protein was extracted from the pellets using 6 M urea lysis buffer [containing 6 M urea, 0.1% Triton X-100, 10 mM Tris (pH 8.0), 1 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM EGTA, and 150 mM NaCl] with protease

inhibitor cocktail. Protein concentrations were determined by bicinchoninic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Tight Junctional Protein Analysis

After protein extraction, microvasculature samples (20 μg) were resolved on a 4-12% Tris-glycine gel (Novex, San Diego, CA) for 90 min at 125 V and transferred to a polyvinylidene difluoride membrane for 40 min at 240 mA. Polyvinylidene difluoride membranes were blocked in Tris-buffered saline (141 mM NaCl, 10 mM Tris-base, and 0.1% Tween 20) with 5% nonfat milk for 4 h. Blots were incubated with primary antibody at room temperature for 2 h, rinsed with Tris-buffered saline with 5% nonfat milk for 1 h, and incubated with secondary antibody for 1 h. Blots were developed using enhanced chemiluminescence (ECL+, Amersham, Springfield, IL) and analyzed using Scion image software.

Immunoprecipitation and Immunoblotting

Isolated microvessel homogenates were analyzed for expression of occludin and ZO-1. Immunoprecipitation studies were performed to determine ZO-1 and occludin interactions with other tight junctional and cytoskeletal proteins. In brief, 100 μg total protein was diluted 10-fold with lysis buffer without urea, combined with 5 μg anti-occludin or anti-ZO-1, and incubated overnight at 4°C. The next day, 50 μl of rec-protein G Sepharose beads (Zymed; San Francisco, CA) were added. Samples were incubated for 4 h at 4°C, pelleted, washed twice with 1 M urea buffer, and washed once with 10 mM

Tris (pH 8.0). Samples were resuspended in Laemmli sample buffer and heated to 96°C for 10 min before electrophoresis.

Microvessel samples (20 μg) and immunoprecipitants were resolved on 4-12% Tris-glycine gels (Novex; San Diego, CA) for 90 min at 125 V and transferred to a polyvinylidene difluoride (PVDF) membrane for 30 min at 240 mA. Gelcode blue (Pierce) was used to stain gels and ensure proper protein loading. PVDF membranes were blocked in Tris-buffered saline (TBS) (141 mM NaCl, 10 mM Tris base, and 0.1% Tween 20) with 5% nonfat milk for 4 h. Blots were incubated in primary antibody at room temperature for 2 h, rinsed with TBS with 5% nonfat milk for 1 h, and incubated with secondary antibody for 1 h. Blots were developed using enhanced chemiluminescence (ECL+; Amersham Life Science Products) and analyzed using Scion image.

Statistical Analysis

Statistical significance ($\alpha = 0.05$) for differences in R_{brain} and protein expression of occludin, ZO-1, and immunoprecipitants was determined by one-way ANOVA followed by Newman-Keuls post hoc test. Data are expressed as means ± SE.

Results

Tight Junctional Protein Analysis

Tight junction protein expression is altered during peripheral inflammation. Western blot analysis of the integral protein occludin (Figure 5.1A) showed that it was significantly decreased in the λ-carrageenan and CFA treatment groups (56.9 ± 13.6 and 26.9 ± 10.8% of control, respectively). However, the accessory protein ZO-1 (Figure 5.1B) demonstrated a significant ($P < 0.01$) increase in ZO-1 protein expression in all three inflammation groups compared with control (formalin, 158.9 ± 1.9%; λ-carrageenan, 239.3 ± 8.3%; and CFA, 187.1 ± 4.4%). The cytoskeletal protein, actin (Figure 5.1C) showed no significant difference in expression between the formalin and control groups; however, there was a significant ($P < 0.01$) increase in expression in both the λ-carrageenan and CFA groups compared with control (299.5 ± 38.4 and 261.7 ± 29.7%, respectively). The integral protein, claudin-1 (Figure 5.1D) was present in the rat microvascular endothelium, but expression was unchanged in all inflammatory pain models compared with control.

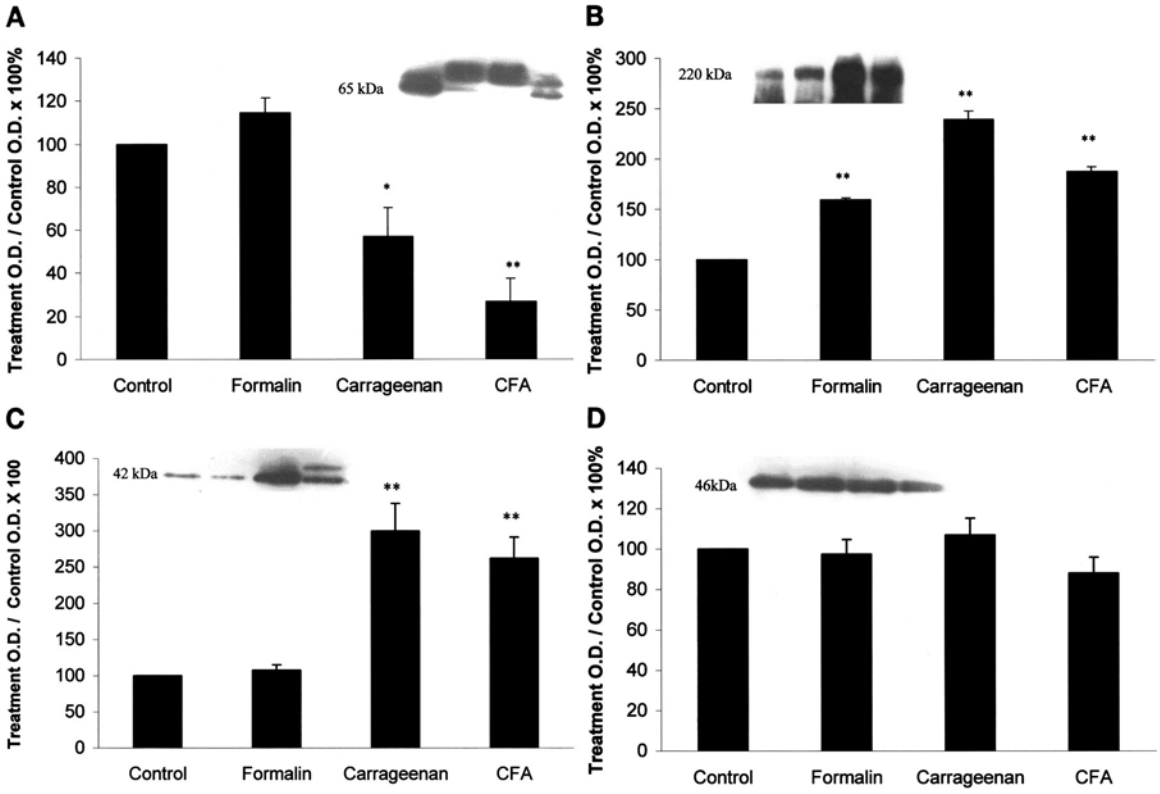

Figure 5.1 Western blot analyses indicate that expression of tight junctional proteins can be altered during peripheral inflammation. *A*: the integral protein occludin was significantly decreased in the λ-carrageenan and CFA inflammatory pain models (56.9 ± 13.6 and 26.9 ± 10.8% of control, respectively). *B*: ZO-1 expression was significantly increased in all 3 inflammatory pain models compared with control (formalin, 158.9 ± 1.9%; λ-carrageenan, 239.3 ± 8.3%; and CFA, 187.1 ± 4.4%). *C*: no differences were seen in actin expression between the formalin and control groups; however, there was a significant difference in actin expression in both the λ-carrageenan and CFA inflammatory pain models compared with that in control (299.5 ± 38.4 and 261.7 ± 29.7%, respectively). *D*: claudin-1 expression was unchanged in the 3 inflammation groups compared with the control. Each bar represents the mean ± SE (n = 3 rats/group). *Insets*: representative Western blots. *Lane 1*, control; *lane 2*, formalin; *lane 3*, λ-carrageenan; *lane 4*, CFA. Statistical significance was determined using one-way ANOVA followed by Newman-Keuls post hoc test. *$P < 0.05$, **$P < 0.01$ vs. control.

Immunoprecipitation and Immunoblotting of Tight Junction Proteins

Western blot analyses indicated an alteration in expression of tight junctional proteins after λ-carrageenan induced-inflammatory pain. Figure 5.2 shows changes in occludin expression after 0- to 72-h treatments. Total occludin expression was significantly reduced at 1, 3, 6, 12, and 48 h [62 ± 7%, 39 ± 10%, 26 ± 3%, 63 ± 8%, and 38 ± 6% of control (0 h), respectively]. Figure 5.2 also shows occludin migrates as two bands, referred to as α and β (Antonetti et al., 1999). During the initial phase of inflammation (0-6 h), the decrease in occludin expression was primarily due to a decrease in the β-band. The second phase (12-72 h) showed a decrease in the α-band with a concomitant increase in β-band expression; an exception was at 48 h, where both α- and β-bands decreased. Table 5.1 illustrates the percent difference in occludin expression in the α- and β-bands from 1 to 72 h compared with the control (0 h).

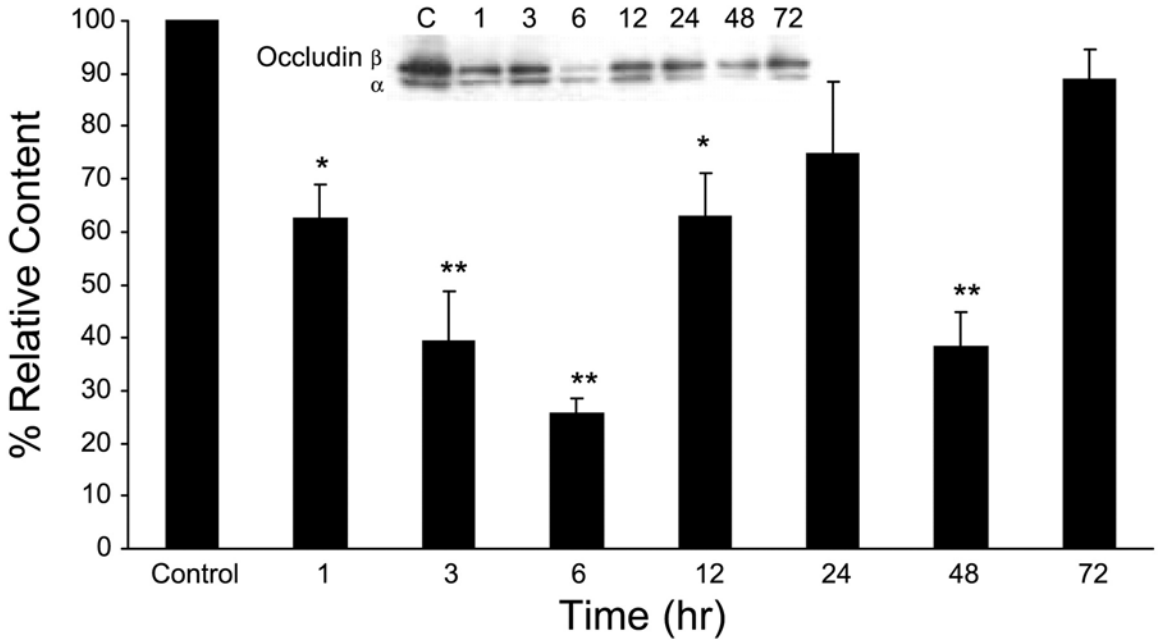

Figure 5.2 Western blot analyses indicate alterations in occludin expression after λ-carrageenan-induced inflammatory pain. Total occludin expression was significantly reduced at 1, 3, 6, 12, and 48 h (62 ± 7%, 39 ± 10%, 26 ± 3%, 63 ± 8%, and 38 ± 6%, respectively) compared with the control (0 h). *Inset*, representative blot image showing occludin migrating as two distinct bands, referred to as α and β. Statistical significance was determined using one-way ANOVA, followed by Newman-Keuls post hoc test. **P* < 0.05 and ***P* < 0.01 versus control.

Table 5.1 Percent relative decrease in occludin expression in the α- and β-bands compared with controls after induction of λ-carrageenan inflammatory pain

Time	α	β
1 h	58 ± 6†	21 ± 11
3 h	51 ± 2†	24 ± 9
6 h	93 ± 3†	73 ± 7†
12 h	24 ± 4*	45 ± 4†
24 h	26 ± 8*	74 ± 7†
48 h	75 ± 7†	92 ± 3†
72 h	17 ± 7	81 ± 8†

Values represent means ± SE; $n = 3$. Statistical analysis was determined using one-way ANOVA, followed by Newman-Keuls post hoc test. * $P < 0.05$ and † $P < 0.01$, significantly different compared with controls (0 h).

Figure 5.3 shows the alterations in ZO-1 expression during 0-72 h of λ-carrageenan-induced inflammatory pain. ZO-1 was significantly increased ($P < 0.01$) at 1, 3, and 6 h [377 ± 76%, 235 ± 17%, and 217 ± 25% of control (0 h), respectively] and returned to control expression levels by 12 h.

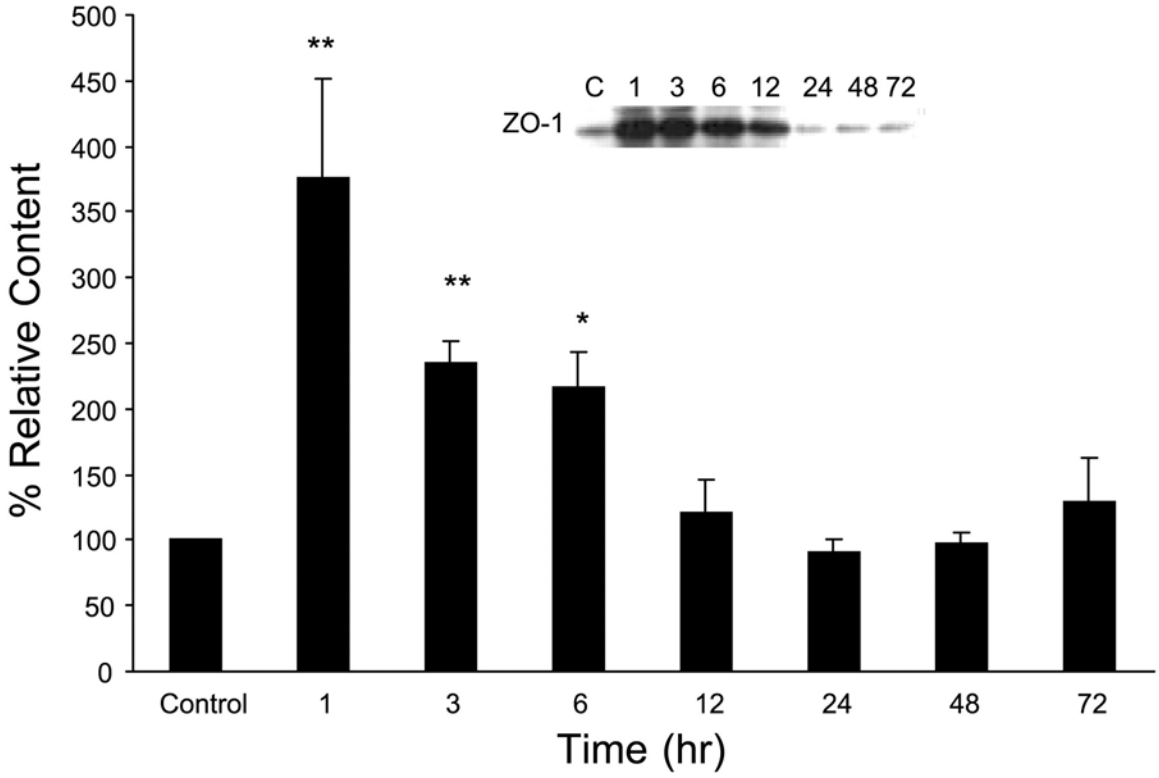

Figure 5.3 Western blot analyses indicate alterations in zonula occludens (ZO)-1 expression after λ-carrageenan-induced inflammatory pain. ZO-1 expression was significantly increased at 1, 3, and 6 h (377 ± 76%, 235 ± 17%, and 217 ± 25%, respectively) compared with the control (0 h). *Inset*, representative blot image showing ZO-1 migrating at 220 kDa. Statistical significance was determined using one-way ANOVA, followed by Newman-Keuls post hoc test. *$P < 0.05$ and **$P < 0.01$ versus control.

Table 5.2 depicts the coimmunoprecipitation of associated tight junctional and cytoskeletal proteins with occludin and ZO-1. Occludin, ZO-2, and actin precipitated with ZO-1. ZO-1 association with ZO-2 increased during the period from 1 to 24 h, whereas ZO-1 association with actin (1-24 h) and occludin (1-6 h) decreased. Results also indicate that ZO-2 did not show a significant change in association with occludin and actin did not immunoprecipitate to any detectable amount with occludin.

Table 5.2 Percent change in protein expression compared with control of proteins immunoprecipitated with ZO-1 or occludin

ZO-1 %Change							
	1 h	3 h	6 h	12 h	24 h	48 h	72 h
Actin	(10 ± 3)	(63 ± 5)†	(48 ± 9)†	(63 ± 6)†	(50 ± 11)†	29 ± 10*	98 ± 5†
ZO-2	53 ± 10†	28 ± 4†	74 ± 12†	57 ± 5†	43 ± 11*	(7 ± 2)	(2 ± 7)
Occludin	(24 ± 7)*	(47 ± 10)†	(15 ± 3)*	4 ± 4	16 ± 4*	10 ± 7	(2 ± 4)

Occludin %Change							
	1 h	3 h	6 h	12 h	24 h	48 h	72 h
Actin	ND	ND	ND	ND	ND	ND	ND
ZO-2	10 ± 5	6 ± 3	3 ± 3	(7 ± 2)	10 ± 4	9 ± 5	3 ± 3

Values are means ± SE; $n = 4$. Values in parentheses indicate a percent decrease in expression compared with controls (0 h); values not in parentheses indicate a percent increase in expression compared with controls. ZO-1, zonula occludens-1; ND, not detected. Statistical analysis was done using one-way ANOVA, followed by Newman-Keuls post hoc test. $^{*}P < 0.0.5$ and $^{\dagger}P < 0.01$, significantly different compared with controls.

Discussion

Tight Junctional Protein Analysis

Currently much advancement has been made in understanding the molecular structure of endothelial cell TJs. However, much less is known about the regulation of the molecular structure under physiological and pathophysiological conditions. Through Western blot analysis proteins can be monitored for changes in expression over a time course. It is already known that tight junction assembly and function can be modulated by a number of signaling molecules, including cAMP, Ca^{2+}, G proteins, phospholipase C, diacylglycerol, small GTP-binding proteins, and protein kinase C (Gonzalez-Mariscal et al., 1985; Balda et al., 1996; Joh et al., 1997; Mullin et al., 1998; Saha et al., 1998).

Tight Junctional Protein Analysis: Occludin

In this study we were able to demonstrate the integral protein occludin showed a dramatic decrease in expression in the λ-carrageenan (3 h) and CFA (3 days) groups (Figure 5.1A). Although occludin has been shown to be not necessary for tight junction formation (Morita et al., 1999), a previous study (Balda et al., 2000) associated the presence of occludin with increased TEER. Previously, it was reported that the inflammatory mediator interleukin-1β decreased occludin expression and increased BBB permeability (Bolton et al., 1998), thus further supporting the results we observed.

The maintenance and assembly of tight junctions under physiological conditions is dependent upon phosphorylation (Nigam et al., 1991; Sakakibara et al., 1997; Wong,

1997). Occludin has several residues capable of being phosphorylated: serine, threonine, and tyrosine residues (Singer et al., 1994; Staddon et al., 1995; Sakakibara et al., 1997). In fact, occludin has a high concentration of glycine and tyrosine (~65%) in the first extracellular loop (Ando-Akatsuka et al., 1996), making it very susceptible to phosphorylation. Studies of occludin and phosphorylation have shown that excessive tyrosine phosphorylation is associated with an increase in epithelial and endothelial cell transcellular permeability (Staddon et al., 1995; Gloor et al., 1997). Further supporting this are studies in diabetic retinopathy where vascular endothelial growth factor stimulated rapid tyrosine phosphorylation of occludin and ZO-1 that led to a 35% decrease in occludin and increased vascular permeability (Antonetti et al., 1999; Antonetti et al., 1999). Unregulated phosphorylation via tyrosine phosphatase inhibition has led to increased tight junctional permeability in human umbilical vein endothelial cells as well as occludin proteolysis (Wachtel et al., 1999). Thus, it appears that BBB permeability is dependent upon maintaining strict control over

Tight Junctional Protein Analysis: Zonula Occluden-1

ZO-1 is an important structural protein in the formation of tight junctional cytoplasmic plaques (Sheth et al., 1997) with phosphorylation sites on serine, threonine, and tyrosine residues (Singer et al., 1994; Staddon et al., 1995; Balda et al., 1996; Collares-Buzato et al., 1998). In this study, we observed an increased expression of ZO-1 in both short-term and long-term inflammatory models [λ-carrageenan (3 h) and CFA (3 days)] (Figure 5.1B), which is not what we observed with occludin. However,

previous studies confirm that unlike occludin, excessive phosphorylation of ZO-1 has not been shown to decrease expression (Wachtel et al., 1999), rather results have been variable. Investigations with Madin Darby canine kidney cells showed that increased tyrosine phosphorylation (via protein phosphatase inhibitors) of ZO-1 in addition to several other junctional proteins had decreased TEER (Ratcliffe et al., 1999). In subconfluent human epidermoid carcinoma cells (A431), stimulation of ZO-1 tyrosine phosphorylation by epithelial growth factor resulted in movement from a diffuse cytoplasmic location to the plasma membrane (Van Itallie et al., 1995). Thus, while the increase in expression may be due to a transcriptional increase in ZO-1, it may more likely be due to a change in phosphorylation states as a cellular compensatory mechanism recruit more tight junctional proteins from the cytoplasm to the plasma membrane.

Tight Junctional Protein Analysis: Actin

Like ZO-1, actin also showed increased expression after peripheral inflammation (Figure 5.1C). This may be due to the tight association of ZO-1 with actin filaments. In a previous study (Blum et al., 1997) a reorganization of the cytoskeletal architecture was observed after pathological insult. Thus, the increased expression of actin, in conjunction with its high association with ZO-1, suggests a reorganization of the cytoskeleton to maintain BBB tight junctional integrity.

Tight Junctional Protein Analysis: Claudin

Claudin is considered to be the major protein involved in maintaining tight junctional integrity (Furuse et al., 1998; Van Itallie and Anderson, 2004). It is embedded in the plasma membrane as dimerized strand and forms a seal with homotypic adhesions with claudins of the adjacent cell (Furuse et al., 1999). The integrity of the tight junction varies dependently on the claudin species involved and their combinations and is dependent upon the strength of the interactions between claudin strands (Furuse et al., 1999). Recent studies have identified at least 24 different claudin genes with different tissue-specific expression and barrier functions (Tepass, 2003). Brain endothelial cells have been shown to at least express claudin-5 and -12 (Matter and Balda, 2003). Thus, a family of claudin may be a target for modifying the absorption of drugs. A recent study by Kondoh et al. using a C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) as a modulator of claudin-4 was able to show an increase in dextran permeability specifically in rat jejunum (Kondoh et al., 2005).

At the time of this study, not much was known about what claudin species were expressed in cerebral endothelial cells. Thus, in this study, we examined the expression of claudin-1 and determined its presence in the capillaries of the BBB (Figure 5.1D). This study was one of the first to identify a claudin subtype in cerebral endothelial cells of the rat. Claudin-1 expression was located at 46 kDa, indicating its presence in the dimerized form. Also, the unchanged expression of claudin-1 after inflammation suggests BBB tight junctions remained grossly intact during inflammation, which supports the general belief that claudin is the major protein involved in maintaining tight junctional integrity (Furuse et al., 1999; Van Itallie and Anderson, 2004). Recent evidence have also shown

the expression of claudins-5 and -12 in brain endothelial cells. It is possible that while claudin-1 expression did not change, expression of the other claudins could have as a compensatory mechanism as suggested by a recent study with claudin-5 knockout mice (Nitta et al., 2003). Future studies need to be done to examine the expression of claudin-5 and -12.

Tight Junctional Protein Analysis: Conclusion

We have shown that inflammatory-mediated pain states alter both the functional and molecular properties of the BBB. In fact, we demonstrate a correlation between increased BBB permeability and altered expression of important tight junctional proteins. These results suggest that peripheral inflammation stimulates reorganization of tight junctions, leading to increased paracellular diffusion. While occludin and ZO-1 play critical roles in regulating permeability changes at the tight junctions, the exact mechanism(s) remains unclear. Previous reports suggest that these proteins are regulated by their phosphorylation states and play an important role in how tight junctions alter permeability during various immune-mediated pain states. This is the first report of peripheral inflammation inducing alterations in tight junctions and increasing permeability of the BBB. These inflammatory-mediated BBB changes may have a significant impact on the delivery of therapeutic agents to the brain. Clinical dosing regimens during chronic inflammatory pain will need to be reevaluated in light of these new findings.

0 – 72 h Tight Junctional Protein Analysis

After the initial studies above we then examined the expression of tight junctional proteins, ZO-1 and occludin over a time course to determine whether the biphasic time course increase in BBB paracellular permeability (Chapter 2; Figure 2.3) was correlated with alterations in tight junctional structural integrity.

0 – 72 h Tight Junctional Protein Analysis: Zonula Occluden-1

We investigated ZO-1 expression over the time course (0-72 h) of λ-carrageenan-induced inflammatory pain. ZO-1 expression was increased during the first phase of the inflammatory process (1-6 h) and returned to basal levels by 12 h (Figure 5.3). ZO-1 expression is not altered at 48 h, although BBB permeability increased. Previous studies have shown that ZO-1 is phosphorylated on tyrosine and serine/threonine residues (Singer et al., 1994; Staddon et al., 1995; Balda et al., 1996), but the effect of phosphorylation on tight junctional physiology remains unclear. Several studies (Antonetti et al., 1999; Wachtel et al., 1999; Atkinson and Rao, 2001) have shown that tyrosine phosphorylation of ZO-1 increases paracellular permeability. However, other studies indicate tyrosine phosphorylation of ZO-1 is important for tight junction assembly and establishment of barrier resistance (Tsukamoto and Nigam, 1999; Meyer et al., 2001). These differing studies reflect the complexity of formation and maintenance of tight junctions and may be a result of diverse signaling pathways.

To further evaluate the possible role of tight junctional proteins in reorganization of the tight junction after an inflammatory insult, we immunoprecipitated with antibodies

to ZO-1 and occludin and probed for associated proteins. Table 5.2 shows the association between ZO-1 and actin significantly decreased at 3, 6, 12, and 24 h but was significantly increased at 48 and 72 h. In contrast, the association between ZO-1 and ZO-2 was significantly increased at 1, 3, 6, 12, and 24 h and decreased at 48 and 72 h. These findings are very interesting and bring up many questions regarding the dynamics of tight junction reorganization. As BBB permeability increases, ZO-1 appears to be less tightly associated with actin and more tightly associated with ZO-2, perhaps indicating a disruption between the tight junction scaffold and the cytoskeleton.

0 – 72 h Tight Junctional Protein Analysis: Occludin

Occludin plays a dynamic, functional role in regulating tight junction integrity during λ-carrageenan-induced inflammation. Numerous phosphorylation sites allow occludin to rapidly respond to environmental stimuli (Antonetti et al., 1999; Tsukamoto and Nigam, 1999; Sheth et al., 2000; Hirase et al., 2001). Our data demonstrated time-dependent changes in occludin expression from 0 to 72 h, with statistically significant reductions in occludin expression at the same time as increased BBB permeability was observed (i.e., 1, 3, 6, and 48 h) (Figure 5.2). As has been previously shown, reductions in occludin expression decrease paracellular permeability, resulting in an increased flux between BBB endothelial cells (Wachtel et al., 1999; DeMaio et al., 2001).

Phosphorylation of occludin regulates tight junction function by redistributing occludin from the cytoplasm to the lateral surface of the plasma membrane (Farshori and Kachar, 1999; Andreeva et al., 2001). Phosphorylation of occludin occurs at both tyrosine

and serine/threonine sites and correlates with permeability changes in existing tight junctions and assembly of new tight junctions (Triguero et al., 1990; Fanning et al., 1998). Two migrating bands recognized by anti-occludin antibodies, referred to as α and β by Antonetti et al. (Antonetti et al., 1999), demonstrated evidence for a change in occludin posttranslational modification. The α-band migrates at 60 kDa, and the β-band migrates at 62 kDa. In this study, occludin migrated most strongly in the β-band, which has been characterized as posttranslationally modified (Antonetti et al., 1999). As Table 5.1 depicts, the loss of occludin expression occurs primarily in the β-band at 1, 3, 6, and 48 h. During the latter portion of the time course, most of the decreased expression occurred in the α-band. During periods of increased BBB permeability, occludin expression in the β-band decreased, whereas during periods showing improved BBB function, occludin expression decreased primarily from the α-band, suggesting that occludin may redistribute from the α-band to the β-band during reassembly of barrier function.

0 – 72 h Tight Junctional Protein Analysis: Conclusion

These findings support the initial tight junctional protein analysis studies and provide further insight into what molecular changes are occurring to TJ proteins over time. We initially showed the λ-carrageenan-induced inflammatory pain model elicited a biphasic increase in BBB permeability, with an initial phase occurring from 1 to 6 h and a second phase at 48 h (Figure 2.3). Furthermore, changes in BBB permeability correlated with changes in the tight junction occludin expression and modified protein-protein

interactions between ZO-1 and occludin, ZO-2, and actin. The exact mechanisms by which these changes occur are still unknown; however, evidence clearly supports the idea that changes in tight junctional organization play a role in increased BBB paracellular permeability. The findings suggest that the λ-carrageenan-induced inflammatory pain model produces alterations in BBB function that may affect CNS homeostasis and have important clinical ramifications concerning therapeutic drug delivery and drug dosing regimens during pain.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

Currently, there is a growing body of research characterizing the BBB under normal physiological conditions; however, little is known about BBB regulation under pathophysiological conditions, such as inflammatory pain. This dissertation demonstrates that peripheral inflammatory pain has a significant influence on the BBB both functionally in terms of permeability and structurally in terms of TJ protein expression and regulation.

Chapter 2 investigated how the ability of the BBB to maintain brain homeostasis may be compromised by inflammatory pain. Using three established models of inflammatory pain (acute: formalin; short term: λ-carrageenan; and long term: CFA) with an *in situ* brain perfusion model of BBB permeability, we were able to investigate the BBB delivery of a normally membrane impermeant marker, [^{14}C] sucrose and a commonly used clinical drug for inflammation/pain, codeine. Initial studies with sucrose showed all three inflammatory pain models compared to control had a significantly (p<0.01) higher distribution of sucrose into the brain (formalin, 67.3%; λ-carrageenan, 62.5%; and CFA, 87.6%) at their maximal point of inflammation. This indicated there was a change in vascular volume and/or an increase in BBB permeability (Fig. 2.2).

To elucidate the cause of these permeability increases, capillary depletion and cerebral blood flow studies were performed. These studies can help determine whether the increased BBB permeabilities were due to increased paracellular diffusion and not changes in vascular space or vascular trapping. Results showed that the change in BBB

permeability was most likely due to increased paracellular diffusion. Also, because [^{14}C] sucrose is a membrane impermeant marker, paracellular diffusion is its primary mode of delivery across the BBB. This further supports the permeability alterations were most likely due to disruptions in BBB endothelial cell tight junctions leading to an increase in paracellular diffusion.

These initial studies established peripheral inflammatory-mediated pain states increased BBB permeability and altered paracellular diffusion at peak inflammation. Knowing this, we next wanted to study the effects over a time course. With a time course study we could better understand functional regulation of the BBB in time. Thus, we chose to investigate the effects of λ-carrageenan-induced inflammatory pain on the functional integrity of BBB tight junctions over 0-168 h of inflammation. λ-Carrageenan-induced inflammation elicited a biphasic increase in [^{14}C] sucrose BBB permeability at 1-6 h and at 48 h (Figure 2.3). The increase seen in this study from 1 to 6 h was consistent with our previous findings at 3 h and demonstrated the maximal increase in BBB permeability coincided with maximal inflammatory response (Winter et al., 1962; Parente et al., 1979). Capillary depletion studies again proved this result was not due to vascular trapping and more likely due to increased paracellular diffusion.

There are numerous therapeutic agents used in the management of illnesses with a peripheral pain component with molecular masses similar to that of sucrose (342 Da), such as morphine (285 Da), codeine (300 Da), acetaminophen (150 Da), methotrexate (454 Da), fluoxetine (320 Da), amitripyline (278 Da), and cyclobenzaprine (276 Da), whose transport into the CNS may be different than seen in healthy individuals. Many of

these compounds have narrow therapeutic ranges and serious neurotoxicity, thus slight perturbations in the BBB as already demonstrated with [^{14}C] sucrose are highly detrimental. This concern led us to the next study investigating the effect of inflammatory pain on the BBB drug delivery of a clinically used drug, codeine.

Codeine is a moderate mu opioid receptor agonist and is prescribed for its antitussive and antinociceptive properties (Goodman et al., 2001). Its action is centrally-mediated thus any breakdown in BBB integrity can lead to significant side-effects (aside from increased antinociception) such as respiratory depression, nausea, vomiting, dizziness, mental clouding, addiction, and acute tolerance (Goodman et al., 2001). In this study we observed increases in codeine permeability at 3 and 48 h (Figure 2.4) which correlated with previous [^{14}C] sucrose BBB permeability results (Figure 2.3). This is the first known observation that peripheral inflammation results in greater analgesic drug uptake to the brain.

In Chapter 3 we knew inflammatory pain increased the BBB permeability of codeine so we were interested in examining what this meant functionally in terms of the antinociceptive effect of codeine. We investigated the potential of codeine to elicit antinociception at the points of increased [^{14}C] sucrose permeability, 3 and 48 h. Nociceptive study results showed that rats treated with codeine experienced enhanced antinociception under λ-carrageenan induced inflammatory pain as compared to saline controls, demonstrating a potential increase in CNS effect during peripheral inflammatory pain (Figure 3.2). The results from increased [^{3}H] codeine permeability

indicating a BBB compromise from λ-carrageenan induced inflammatory pain, combined with enhanced codeine analgesia, suggest a greater CNS dose of codeine.

In Chapter 4, to address concerns of a direct effect of the inflammatory agents we had been using in our studies we investigated the influence of the three inflammatory agents on the functional and structural integrity of the blood-brain barrier. Cellular cytotoxicity and paracellular permeability were assessed *in vitro* using primary bovine brain microvascular endothelial cells exposed to formalin, λ-carrageenan, or complete Freund's adjuvant for 1, 3, or 72 h, respectively. Results showed that only the highest concentration (0.025%) of formalin produced a decrease in cell viability (approximately 34%) and a significant increase in cell permeability to [^{14}C] sucrose at 120 min (approximately 137%). Brain perfusion using female Sprague-Dawley rats showed no difference in paracellular permeability to [^{14}C] sucrose for any inflammatory agent. Western blot analyses were performed on isolated rat brain microvessels to assess the structural integrity of blood-brain barrier tight junctions. Results indicate that expression of zonula occludens-1, occludin, claudin-1, and actin remain unchanged following intravenous exposure to inflammatory agents. This study confirms that changes seen at the blood-brain barrier following a peripheral inflammation are due to physiological responses to the given inflammatory agent and not to any direct interaction between the inflammatory agent and the brain microvasculature.

Recognizing inflammatory pain increased BBB permeability functionally, we next investigated why this was happening. In other words, how was the BBB being affected structurally and molecularly. We were interested in elucidating what was

happening at the level of the TJ proteins to cause these BBB perturbations. Chapter 5 describes how we set out to investigate this and our findings.

Western blot analyses of TJ protein expression during maximal points of inflammation of all three inflammatory models (formalin; λ-carrageenan; and CFA) showed varying degrees of altered tight junctional protein expression. Occludin significantly decreased in the λ-carrageenan and CFA-treated groups. Zonula occluden-1 expression was significantly increased in all pain models. Claudin-1 protein expression was present at the BBB and remained unchanged during inflammation. (This study was one of the first to identify a claudin subtype in cerebral endothelial cells of the rat.) Actin expression was significantly increased in the λ-carrageenan and CFA-treated groups. These results suggest that peripheral inflammation stimulates reorganization of tight junctions, leading to increased paracellular diffusion. This is the first report of peripheral inflammation inducing alterations in tight junctions and increasing permeability of the BBB.

When ZO-1 and occludin were examined over a time course, ZO-1 expression was shown to be significantly increased at 1, 3, and 6 h and returned to control expression levels by 12 h. Total occludin expression was significantly reduced at 1, 3, 6, 12, and 48 h. In addition, there was a modification of protein-protein interactions between ZO-1 and occludin, ZO-2, and actin. These results demonstrate that λ-carrageenan-induced inflammatory pain altered tight junctional protein expression of occludin and ZO-1. Furthermore, the alterations correlate with the previously observed biphasic increase in BBB permeability with the first phase occurring from 1-6 h and the second phase

occuring at 48 h. This suggests BBB cytoarchitecture alterations underlie the BBB permeability changes observed previously. The exact mechanisms by which these changes occur are still unknown; however, evidence clearly supports the idea that changes in tight junctional organization play a role in increased BBB paracellular permeability. These findings suggest that the λ-carrageenan-induced inflammatory pain model produces alterations in BBB function that may affect CNS homeostasis and have important clinical ramifications concerning therapeutic drug delivery and drug dosing regimens during pain (Huber et al., 2001; Huber et al., 2002; Huber et al., 2002; Hau et al., 2004).

This dissertation focused on disruptions in therapeutic drug delivery, antinociception, and tight junctional protein changes under a pathophysiological condition, inflammatory pain. The majority of drug delivery studies have traditionally been done in naïve animals, and thus the results and conditions may not be applicable to the situation in which the patient is in pain. In fact, inflammatory pain may affect a drug's delivery, effectiveness and/or therapeutic index. Because pathophysiological conditions must be considered in assessing drug dosing, this dissertation provides support for planning effective drug delivery regimens. In opioid equianalgesic conversion, numerous factors can affect dosing level, such as the patient's current pain syndrome (Brant, 2001); thus, it should be common practice to initially prescribe based on factors such as the possibility of BBB compromise during inflammatory pain. By ensuring the proper dose for a pained patient, CNS adverse drug effects could be attenuated or avoided. Having a better understanding of BBB structure and function in response to

inflammatory pain will provide further insight into more effective and safer therapeutic approaches to treating inflammatory pain diseases.

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