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MASTERARBEIT

Titel der Masterarbeit

"Effect of peripheral λ-carrageenan-induced inflammation on the blood-spinal cord barrier and the tight junction protein occludin"

Verfasserin

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1. Introduction

The blood-brain barrier (BBB) and the blood-spinal cord barrier (BSCB) provide a separation "fence" between the blood circulation and the central nervous system. They consist of microvascular endothelial cells separated by tight junctions and forming the vascular wall and interact with many different cell types of the nervous system like pericytes, astrocyte endfeet, microglia and neurons. This interaction provides a "neurovascular unit" which protects the susceptible environment of the central nervous system from harmful substances and pathogens out of the blood. Hence, it is essential for the maintainance of fundamental neurological functions. Large and hydrophilic molecules are restricted from passive diffusion through the microvascular endothelial cells and also through the tight junctions between them. The key players of the paracellular diffusion restriction are tight junction proteins, which are assembled in the space between endothelial cells. Many diseases can influence the assembly of tight junction proteins, which can then change the BBB or BSCB properties (Hawkins and Davis 2005; Zlokovic 2008). It is suggested that activation of sensory and nociceptive nerves may lead to the release of mediators which cause the breakdown. Peripheral inflammatory pain has been shown to influence the integrity of the BBB and lead to decreased distribution of the tight junction proteins such as occludin (Huber et al. 2001; Huber et al. 2002). Furthermore, recent findings suggest that peripheral nerve injury induced by constriction or ligations leads to increased permeability of the BSCB and BBB (Beggs et al. 2010). Since afferent nerve fibres project to the spinal cord and their activation may lead to pro-inflammatory mediators, we wanted to investigate the effect of a peripheral inflammation due to λ-carrageenan injection on the BSCB. We examined the distribution of the tight junction protein on endothelial cells and the presence of immunoglobulin G (IgG) in parenchymal tissues. Occludin plays an essential role in the regulation of tight junction permeability in endothelial cells (Hirase et al. 1997) and IgG is not normally found inside the CNS. Furthermore, we studied this effect in both male and female rats. Previous findings showed a difference in the BBB permeability between male and female rats (Bake et al. 2009; Oztas et al. 2001) and therefore, we assumed a different permeability of the BSCB in the λ-carrageenan induced inflammatory pain model.

1.1. The Blood-Brain Barrier

The central nervous system (CNS) is separated from the blood circulation through a very selective barrier at the level of brain microvascular endothelial cells, called blood-brain barrier (BBB). Compared to the barriers between blood and the peripheral nervous system or non-neuronal tissue, the BBB has a very low permeability and restricts large and hydrophilic molecules, pathogens, immune cells, drugs and many other substances which can disturb the sensitive microenvironment of the central nervous system. This is essential for maintaining the basal neurological functions. Neurons need a constant ion concentration to maintain a voltage gradient across their membrane in order to transmit action potentials and electrical signals. Since the ion concentration of the blood can change rapidly, e.g. after ingestion (Abbott et al. 2006), it is essential that the nervous system gets protected from these changes.

The brain microvascular endothelial cells which form the vascular wall interact with the basement membrane and different cells such as pericytes, astrocytes, microglia and neurons (**Figure 1**). They constitute a "neurovascular unit" that is essential for the function of the CNS.

In addition to the protective function, the BBB also affords a passage for some compounds via the paracellular pathway between the endothelial cells or the transcellular pathway through the cells. The supply of nutrients and the efflux of waste products are mediated by the BBB. Gas molecules like oxygen and carbon dioxide and most lipophilic molecules such as steroid hormones and lipid soluble vitamins, can freely diffuse through the BBB. Thus, the passive diffusion of these molecules depends on the concentration gradient, the molecular weight, the lipid solubility and the ionization degree (Scherrmann 2002). The negatively charged surface of brain microvascular endothelial cells prevents the penetration of negatively charged molecules (Cardoso et al. 2010). Hydrophilic nutrients get transported via selective transporters (Hawkins et al. 2002). Glucose represents the most important energy source for the brain and gets transported via glucose transporter-1 (GLUT-1) through the endothelial cells. Furthermore, ATP-binding cassette (ABC) transporters are responsible for the passage of many hydrophilic molecules, such as ions, polysaccharides, amino acids, peptides, vitamins and hormones (Jones and George 2004; Shen and Zhang 2010). Adjacent endothelial cells are separated by tight junctions who are responsible for the regulation of the paracellular transport (see below).

Whereas the microvascular endothelial cells are the key players of the BBB function and properties, the other adjacent cells have a supporting and regulating role (Cardoso et al. 2010). Pericytes are undifferentiated cells and have a direct contact with microvascular endothelial cells. They support the vessels, are responsible for the angiogenesis and also involved in BBB permeability (Persidsky et al. 2006). A reciprocal communication and interaction between pericytes and endothelial cells are essential for vascular development, maturation and microvessel architecture (Choi and Kim 2008; Bell et al. 2010). An age dependent loss of pericytes induces neurovascular dysfunction and BBB disruption. This leads to further accumulation of neurotoxic molecules in the brain potentiating neurodegeneration and neuroinflammation (Bell et al. 2010). The basement membrane surrounds the endothelial cells and pericytes, consists of structural proteins and anchors these cells in place (Persidsky et al. 2006; Cardoso et al. 2010). Inflammations of the CNS can lead to a damaged basement membrane resulting in a disturbed BBB (Garbuzova-Davis et al. 2007a; Garbuzova-Davis et al. 2007b). Astrocytes provide close contact to the endothelial cells of the microvessel wall via their perivascular endfeet and are essential for structure and function of the neurovascular unit. Due to specialized channels they are important for the ion and volume regulation (Abbott et al. 2006). Astrocytes possess glucose transporters in their endfeet and are competent for glycolysis, hence for energy supply (Simard and Nedergaard 2004). Furthermore, they secrete several growth factors like transforming growth factor-β (TGFβ), glial-derived neurotrophic factor (GDNF) and fibroblast growth factor (bFGF) which can influence the BBB function (Igarashi et al. 1999; Abbott et al. 2006). Another glial cell type are microglia which constitute the primary immune effector cells in the nervous system. They are highly active during their resting state and can quickly detect disturbances of the BBB. Inflammations of the nervous system lead to a fast central-mediated activation of microglia (Huber et al. 2006). During vascular injury, microglia assume a shielding role and clear debris via phagocytosis (Nimmerjahn et al. 2005). They also respond to changes in the microenvironment by releasing different immunoregulatory mediators such as pro-and anti-inflammatory cytokines and neurotrophic factors, which can lead to a disruption of tight junction proteins (Poritz et al. 2004). An important regulatory function in the neurovascular unit is also attributed to neurons which can have a direct innervation of microvascular endothelial cells or by neuronal-glial interactions via noradrenergic, serotonergic, cholinergic and GABA-ergic synapses (Hawkins and Davis 2005; Choi and Kim 2008).

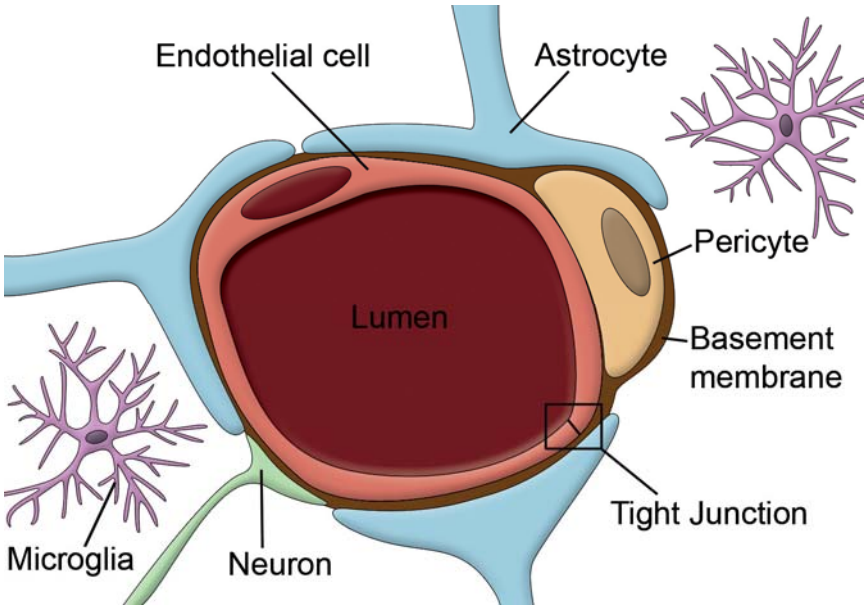

Figure 1: Illustration of the BBB. The endothelial cells form the vascular wall and are surrounded by the basement membrane. They interact with many different cell types, like pericytes, astrocytes, microglia and neurons. This interaction provides a "neurovascular unit" which is essential for the function and protection of the CNS.

1.2. Tight Junctions

The tight junctions between the brain microvascular endothelial cells consist of a complex assembling of tight junction and adhesion molecules, which effectively restrict the paracellular diffusion (**Figure 2**). Compared to tight junctions in peripheral capillaries, the tight junctions in the BBB restrain the movement of ions like Na^+ and Cl^-. This leads to a very high transendothelial electrical resistance in contrast to peripheral blood-nerve barriers (Butt et al. 1990). There are two classes of transmembrane proteins, occludin and claudins, which share similar membrane topography but no sequence homology. Occludin was the first transmembrane molecule of the tight junction discovered (Furuse et al. 1993) and it is exclusively localized at tight junctions of epithelial cells. It is a 60-65 kDa protein and consists of four transmembrane domains with both termini in the cytosol (**Figure 3**). The COOH (C)-terminal of occludin binds to the accessory proteins zona occludens ZO-1 and ZO-2 which link the transmembrane proteins of the tight junction to the actin cytoskeleton. While the C-terminal of occludin modulates the paracellular diffusion and is required for the tight junction barrier function (Chen et al. 1997), the N-terminal has a critical function for the anchoring of occludin and the paracellular migration of neutrophils (Huber et al. 2000).

The functional role of occludin is somewhat mixed. Most studies show occludin as a determinant of tight junction permeability and the occludin expression is often associated with a BBB disruption (Huber et al. 2001; Huber et al. 2002; Brooks et al. 2005; Brooks et al. 2006; McCaffrey et al. 2008). One key study showed that in the tight junctions of Xenopus kidney epithelial cells a synthetic peptide corresponding to the second extracellular domain of occludin can specifically decrease the amount of occludin and lead to an increased tight junction permeability (Wong and Gumbiner 1997). Occludin-deficient mice (Saitou et al. 2000) showed a complex phenotype with chronic inflammation and abnormalities in postnatal growth and sexual behaviour, but the morphological structure and function of the tight junctions were not affected. The results of studies on MDCK I cell cultures suggest that the phosphorylation of occludin is a key mechanism for the regulation and function of this protein (Sakakibara et al. 1997). It has different sites for phosphorylation and the highly phosphorylated occludin form seems to be the functional form of the protein (Sakakibara et al. 1997; Feldman et al. 2005).

In addition to occludin, the transmembrane protein claudin plays an important role in the sealing of the tight junction. The two types claudin-3 and -5 which are found in the cerebral microvascular endothelium and have different functions in maintaining the BBB integrity.

Further proteins of the tight junction are junctional adhesion molecules (JAM)-1. JAM-1 is a transmembrane protein with one extracellular domain and it mediates the interactions attachment of adjacent cell membranes (Hawkins and Davis 2005). It is hypothesized that JAM-1 indirectly mediates the localization of occludin to the tight junction via ZO-1 (Feldman et al. 2005).

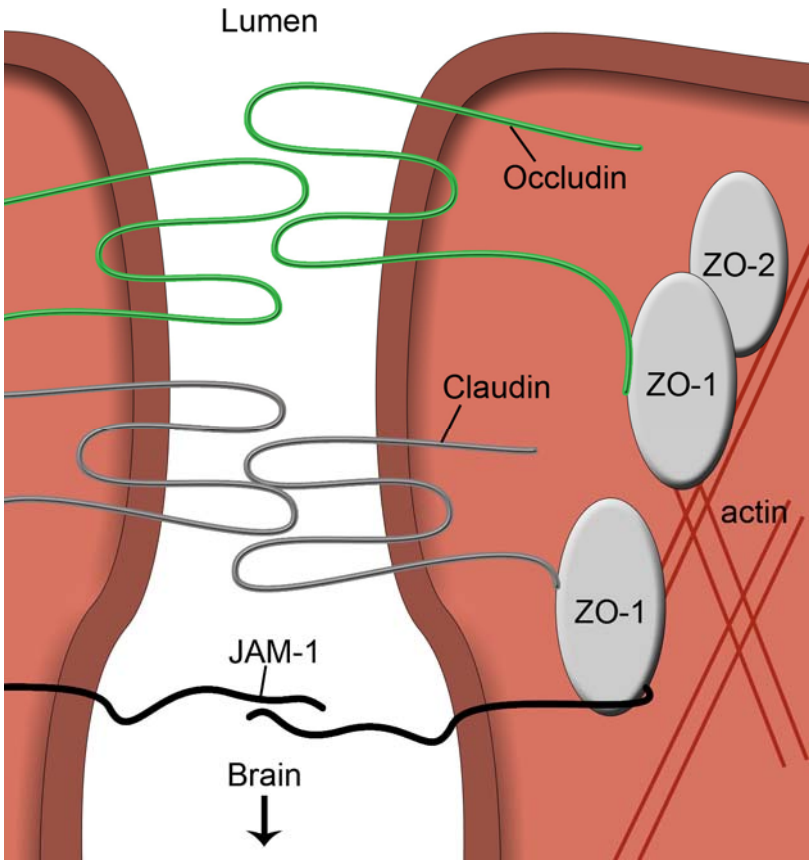

Figure 2: Tight junction formed by two adjacent microvascular endothelial cells in the central nervous system. Occludin and Claudin (the forms Claudin-3 and -5 are present in the BBB and BSCB) are transmembrane proteins and are responsible for the paracellular diffusion barrier. They are linked via zona occluden (ZO)-1 and -2 to the actin cytoskeleton. The transmembrane protein junctional adhesion molecule (JAM)-1 imparts the connection of the adjacent cell membranes. Adapted from Hawkins and Davis (2005).

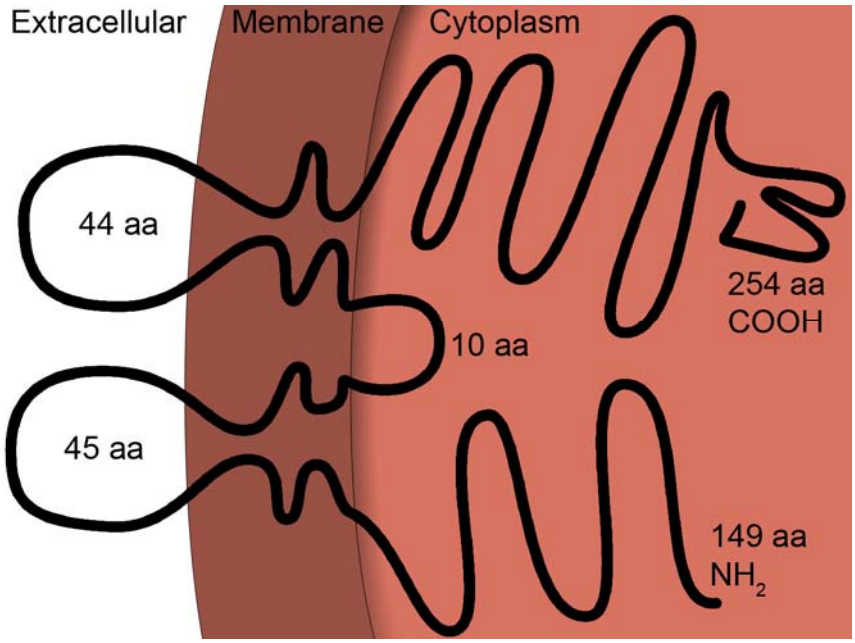

Figure 3: Schematic illustration of occludin structure in the microvascular endothelial cells. aa=aminoacids. Adapted from Feldman and colleagues (2005).

1.3. Blood-Spinal Cord Barrier

Within the nervous system there are different blood-neural barriers including the blood-brain barrier, blood-spinal cord barrier, blood-cerebrospinal fluid barrier, blood-retinal barrier, blood-labyrinth barrier and blood-nerve barrier (Choi and Kim 2008). They differ in several functional and structural properties. The BSCB and the BBB have mostly similar properties with some differences. Pan and colleagues observed a greater permeability to radio-labelled tracers of the thoracic and lumbar region of the spinal cord compared to the brain and the cervical region (Pan et al. 1997a; Pan et al. 1997b). They hypothesized that the vascular space may differ or the presence of the brachial and lumbosacral plexuses increases the spinal paracellular permeability. The BSCB and BBB have different regulatory properties. However, most research assumes a similar function of the BBB and the BSCB and does not specifically examine the BSCB.

1.4. Dysfunction of the blood-brain and blood-spinal cord barrier in pathological conditions

The neurovascular unit is the functional gateway between the nervous system and the blood circulation and gets regulated by different cell types which respond to physiological,

immunological, metabolic and endocrine conditions and requirements. Thus, the BBB and BSCB are influenced by many factors and a wide range of diseases have a direct and indirect impact on the barrier integrity and permeability (Persidsky et al. 2006; Zlokovic 2008). If the BBB permeability is changed during disease, an increased efflux of toxins, immune cells, and pathogens from blood to brain can lead to a damage of the nervous system. In addition, increased delivery of therapeutic drugs into the parenchyma can lead to enhanced side effects of those drugs.

In male patients with Alzheimer's disease, a significant positive increase of the albumin ratio between cerebrospinal fluid and blood was found (Algotsson and Winblad 2007). This indicates a leakier BSCB. Increased BSCB permeability is also a constitutive event in multiple sclerosis, an inflammatory disease of the CNS. Mice that develop experimental autoimmune encephalomyelitis (EAE) – an animal model for investigation of multiple sclerosis (MS) – show a BSCB breakdown at the beginning and peak of the disease (Schellenberg et al. 2007; Fabis et al. 2007). Normal barrier function was restored during disease remission again. This early onset of BBB and BSCB disruption may enable the leakage of inflammatory cells which may contribute to the CNS damage. Similar results were found in an animal model with amyotrophic lateral sclerosis (ALS) (Garbuzova-Davis et al. 2007a; Garbuzova-Davis et al. 2007b), where the increased BSCB permeability plays a primary role in pathogenesis. Cerebral ischemia is characterized by an insufficient oxygen-supply/hypoxia and nutrition supply in the nervous system due to loss of blood flow. Studies with rats that underwent arterial occlusions show increased BBB permeability which correlates with altered tight junction protein (Hatashita and Hoff 1990; Won et al. 2011). This tight junction protein loss may attribute to iron-mediated free radical production (Won et al. 2011). Hypoxic stress under low oxygen-concentration, also with a reoxygenation of 10 min, leads to increased cerebral microvascular permeability and to a decreased expression of non-phosphorylated occludin, whereas the amount of claudin-3, ZO-1 and actin does not change (Witt et al. 2003).

Pathological conditions in the periphery involving pain also lead to impairments of the BBB and BSCB (Wolka et al. 2003). Animal models of neuropathic pain induced by traumatic sciatic nerve injury or ligation of spinal nerves show an alteration in BSCB permeability (Gordh et al. 2006; Gordh and Sharma 2006). Electrical stimulation of nociceptive C-fibres of the sciatic nerve causes a widespread opening of the BSCB (Beggs et al. 2010). This effect can be prevented by a prior application of the local anaesthetic lidocaine (Beggs et al. 2010). Furthermore, pain induced by peripheral inflammation increases the BBB and alters the

expression of tight junction proteins (Huber et al. 2001; Huber et al. 2002; Brooks et al. 2005; Brooks et al. 2006), which again can prevented with a local anaesthetic drug (Campos, 2008).

Drug administration can also have effects on the BBB. Chronic administration of morphine shows an effect on the BBB with a slightly increased occludin expression after 5-day treatment (Yousif et al. 2008). Morphine withdrawal also causes a severe BBB breakdown (Sharma and Ali 2006). It is assumed, that these changes are correlated with withdrawal stress symptoms. Also other stress factors, like surgical pain stress increases the BBB (Öztas et al. 2004).

1.5. Investigation of the blood-brain and blood-spinal cord barrier

In this study we decided to use an animal model with a carrageenan-induced inflammation of the hindpaw to investigate the effects on the BSCB permeability and tight junction protein assembling. In a recently published paper of Beggs and colleagues (2010) altered permeability of the BSCB was shown after chronic constriction injury (CCI), spared nerve injury (SNI), C-fibre stimulation of the sciatic nerve and capsaicin application on the sciatic nerve. These are approaches which directly address nociceptive C-fibres (via electrical stimulation and TRPV1-activation due to capsaicin) and induce neuropathic pain (CCI and SNI). In the next part different approaches for animal models of pain and the investigation of BBB and BSCB permeability and tight junction proteins will be discussed.

1.5.1. Animal models of inflammatory pain

There is a wide range of animal models used for peripheral-induced pain conditions and to investigate the underlying mechanisms of pain (Sandkühler 2009). Peripherally induced inflammation by formalin (Abbott et al. 1995) or Complete Freund's adjuvant (CFA) injection (Brooks et al. 2006; Brooks et al. 2006) leads to an early long lasting hyperalgesia. However, the effect on the BBB is delayed in the CFA model, with a decreased occludin distribution after 3 days of inflammation (Brooks et al. 2006). In the inflammatory pain model with λ-carrageenan hindpaw injection an early long-lasting allodynia beginning 1 hour post-injection has been shown (Campos et al. 2008) but a biphasic and reversible effect on BBB permeability and occludin distribution with an early change after 1 hour and a later effect after 48 hours (Huber et al. 2002). In this study, we used an animal model with carrageenan-induced peripheral inflammation of the left hindpaw to investigate the effect on

the BSCB. This inflammation consists of neurogenic and immunological mechanisms. Early extravasation of neutrophils into the subarachnoid space in the brain was found and studies indicate an enhanced metabolism of arachidonic acid (Di Rosa et al. 1971; Gamache et al. 1986). The arachidonic acid metabolism leads to an increased production of proinflammatory mediators like prostaglandins. Furthermore, release of histamine, 5-HT and kinins is involved in carrageenan-induced peripheral inflammation (Di Rosa et al. 1971). These mediators are released by different cell types in the nervous system and all have an effect on the permeability of the BBB (Abbott 2000). Since afferent neurons from the periphery project to the spinal cord, the effect of carrageenan-induced peripheral inflammation has a likely effect on the BSCB integrity. The effect is assumed to be similar to the effect on the BBB (Pan et al. 1997a; Pan et al. 1997b). Findings suggest differences of carrageenan-induced nociception between male and female rats, with lower pain thresholds in females compared to males (Tall and Crisp 2004). The results of gonadectomised rats (Tall and Crisp 2004) and estrogen receptor knockout mice (Li et al. 2009) indicate the prominent influence of gonadal hormones on sex-related reaction on carrageenan. Furthermore, sex-differences in BBB permeability were found in rats with epileptic seizures, with a higher permeability in female rats (Oztas et al. 1992). Hence, in this study both male and female rats were used to investigate possible sex differences in the BSCB properties.

1.5.2. Investigation of the barrier permeability

To analyse changes in permeability of the BBB and BSCB, it is possible to take advantage of the typical properties of the barrier, such as the restricted diffusion to hydrophilic and large molecules. Therefore, an artificial or endogenous tracer in the blood which usually do not or only barely cross the barrier but cross due to increased permeability can be used to investigate the integrity of the barrier. Common used tracers are sugars like sucrose and mannitol labelled with radionuclides (Huber et al. 2002; Brooks et al. 2005; Brooks et al. 2006; Brooks et al. 2008; Campos et al. 2008; McCaffrey et al. 2008). In contrast to glucose or fructose, they do not get transported from the blood to the brain but appear into the parenchyma only after BBB breakdown. Also radio-labelled therapeutic drugs can be used to investigate their barrier permeability in different disease conditions (Hau et al. 2004). The tracer is added via peripheral injection or during the perfusion of the animal and thus, ends up in the blood circulation and may cross the BBB. The ratio between the radiolabeled isotopes in the perfusate compared to the nervous system is used for the quantification of the

extravasation. The disadvantages of this method are the special permissions and safeguards to work with radioactivity.

Another often used method is the analysis of endogenous blood plasma proteins like albumin or immunoglobulin G. These large immunoproteins are unable to cross the intact BBB and BSCB and can be labelled with immunohistochemistry methods. To visualize the albumin leakage in the nervous system, Evans Blue dye is often used. This dye has a very high affinity to albumin, can be introduced during the perfusion and measured by absorbance spectroscopy. To increase the signal it is also possible to use exogenous Evans Blue albumin, also in combination with other fluorescent tracers like fluorescein sodium (Hawkins and Egleton 2006). In this study we stained the endogenous Immunoglobulin G (IgG) of the rats with immunofluorescence. IgG is a large 150 kDa antibody molecule and can be directly stained with a primary fluorescence-conjugated antibody. We chose this easy and fast method, because it was possible to stain it together with blood vessels and occludin to analyse the extravasations of IgG. This method was also used in other studies to investigate the BBB permeability (Pelegrí et al. 2007; Chen et al. 2008).

1.5.3. Analysis of tight junction proteins

The usual way to quantify proteins is via Western blot analyses. However, this method does not show a potential dislocation of the tight junction protein, what could occur during altering of the barrier without destroying or degrading of the protein. To analyse the distribution of occludin at the endothelial cells, we made immunohistochemical stainings of the endothelial cells and the occludin protein in spinal cord slides. In addition to the analysis of the location of occludin at the tight junction, a further advantage was the direct comparison of a leakier barrier at specific vessels to the expression of occludin. One aim of this study was to investigate, if blood vessels, which have extravasation of IgG, exhibit an altered occludin distribution. Furthermore, using immunohistchemical stained spinal cord slides it is also possible to make a semi-quantification of the occludin protein by analysing the occludin staining at the vessels and count vessels with intact, punctate, diffuse, or lost occludin protein (Morgan et al. 2007).

2. Material and methods

2.1. Antibodies and Chemicals

The following substances were used: λ-carrageenan (Sigma Aldrich 22049), sterile 0.9% saline ("Fresenius" Kabi Austria GmbH, Graz, Austria), normal donkey serum (NDS, Millipore S30), DAPI (Sigma D-9542), mouse anti-occludin (Invitrogen 33-1500), Triton X-100 (Merck), rabbit anti-Von Willebrand Faktor (VWF) (Sigma F3520), guinea pig anti-occludin (Hycult HP9047), Cy3 conjugated donkey anti-Rat IgG (Chemicon AP189C), Cy2 conjugated rabbit anti-mouse (Jackson 315-225-003), Cy3 conjugated donkey anti-rabbit (Chemicon AP182C), AMCA conjugated donkey anti-guinea pig (Jackson 706-155-148), Cy2 conjugated donkey anti-rabbit (Jackson 711-225-152).

2.2. Animals and Treatments

Female and male Sprague-Dawlay rats (150-200 g) were housed in separated cages with 4-6 animals with same sex per cage in an independent animal room (25°C, 12 hours light/dark cycle, with food and water ad libitum). All animal experiments of this study are approved by the Ethics-Committee of the Medical University of Vienna and the Bundesministerium für Wissenschaft und Forschung (Tierversuchantrag: "Sex difference and the blood spinal cord barrier in chronic pain"; Nr.:66.009/0063-II/3b/2011).

Before treatment, rats were anesthetized with a mixture of N_2O, O_2 and 5% Isoflurane. 100 µl 3% λ-carrageenan diluted in 0.9% saline was injected in the plantar surface of the left hind paw or subcutaneous into the back and 100 µl of 0.9% saline was used for the vehicle animals.

2.3. Tissue preparation

For the first experiment to investigate occludin, the animals were sacrificed at 3, 24, 72 hours and 5 days (only in males) after λ-carrageenan injection. Vehicle-treated animals were sacrificed at 24 hours post-injection and animals with a subcutaneous injection after 72 hours. For spinal cord collection, the animals were deeply anesthetized using ether and decapitated. The backbone was dissected at the level of the hips and the spinal cord was removed by cold water propulsion. Thereafter, the lumbar and thoracal regions were dissected, wrapped in chicken muscle, snap-frozen in isopentane and stored at −80°C.

In the second experiment we wanted to analyse the integrity of the BSCB via IgG-staining. Therefore, flushing out of the blood of the animals was necessary. The animals were deeply anesthetized with ether, the common carotid artery was exposed and cannulated for the perfusion. The inferior vena cava was cut to relive pressure. As perfusate 0.1 M PB oxygenated with O_2 and heated to 37°C was used. Following a perfusion of 150 ml using a peristaltic pump (15 $ml \cdot min^{-1}$) to flush all the blood out, the rat was decapitated and the spinal cord was removed by cold water propulsion. The lumbar and thoracal region of the spinal cords were wrapped in chicken muscle and snap-frozen in isopentane before storage at −80°C.

All tissue samples were analyzed by the experimenter unaware of the sample identity.

2.4. Immunohistochemistry

Due to the somatotopical organization of afferent neurons from the rat hindpaw, the L5 region of the spinal cord was analysed first (**Figure 4A**). The identification of the regions is possible on the basis of the shape of the grey matter (**Figure 4B**). Fourteen-µm-thick slices of the L5 region were cut transversally in the kryomikrotom (Leica CM 3050S) at −20°C. They were placed up at microscope slides, dried for 1 hour at room temperature and stored at −20°C until immunolabeling. Moreover, slices from the thoracal region (T1-T3) were cut transversally and stained to see if the inflammation has a widespread effect on the IgG extravasation in the spinal cord.

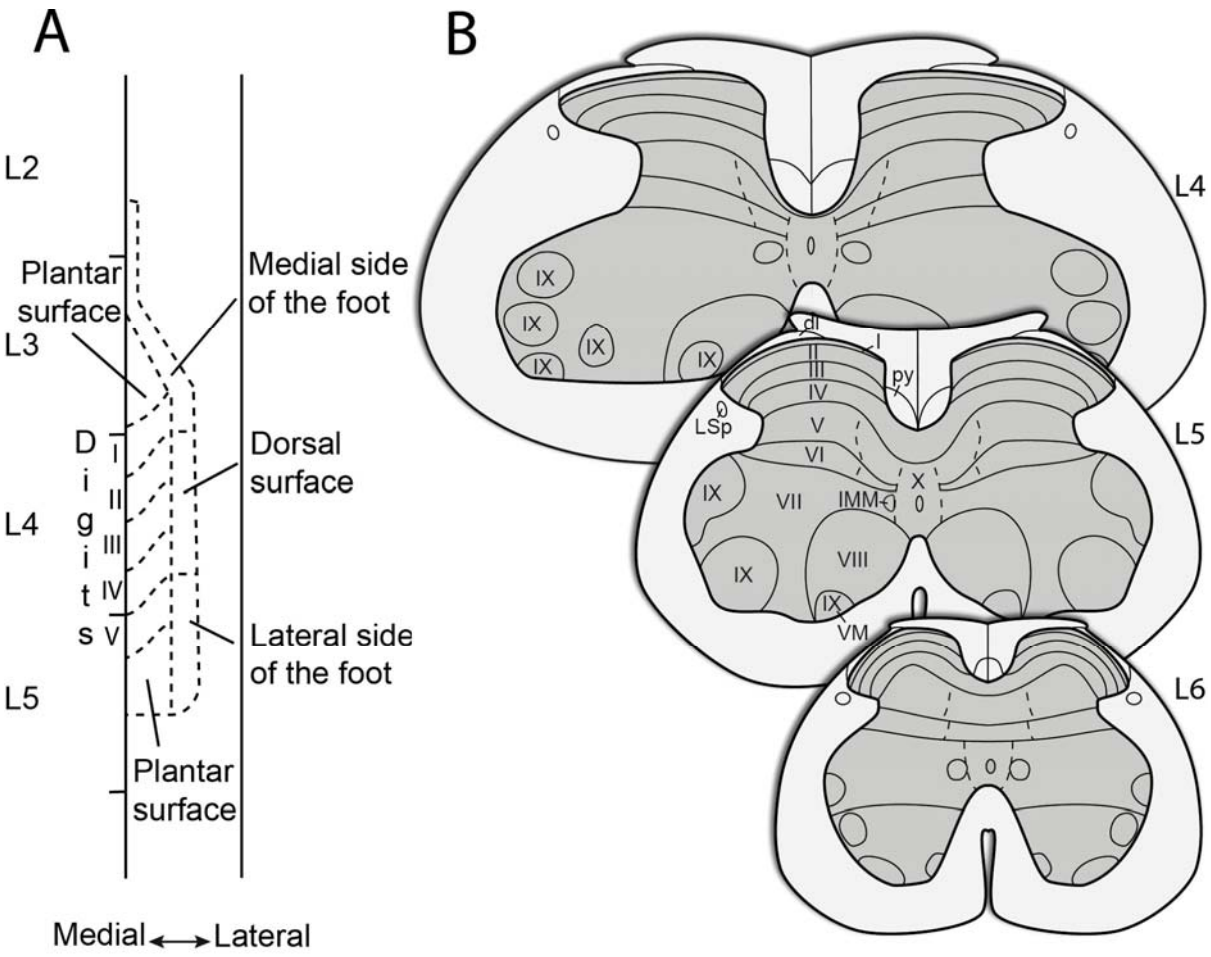

Figure 4: A: Schematic map of lamina II (dorsal view), describes density centers of projections from the different parts of the rat's hind paw skin. Dashed lines indicate overlap. **B**: Schematic illustration of transverse sections of the L4 to L6 regions of the rat's spinal cord. I-X: cytoarchitectonic laminae; IMM: intermediomedial nucleus; LSp: lateral spinal nucleus; py: pyramidal tract; dl: dorso-lateral fasciculus; VM: ventromedial nucleus. (Illustrations are adapted from George Paxinos "The Rat Nervous System" (1994); B: originally illustrated by Molander et al. 1984).

2.4.1. Occludin/Von Willebrand Faktor Staining

After drying for 1 hour at room temperature slides were fixed in 100% ethanol for 30 min at 4°C and then placed in acetone for 3 min at −20°C. Thereafter, the slides were washed once with PBS and blocked with 5% NDS (diluted in PBS) at room temperature in a wet chamber for 30 min. The sections were incubated in a wet chamber with primary mouse anti-occludin (1:1000 dilution in 5% NDS) and rabbit anti-Von Willebrand Faktor (VWF) (1:4000 dilution in 5% NDS) for 1 hour at room temperature. Afterwards, the sections were washed with PBS three times for 5 min each time and incubate in the wet chamber with the secondary antibodies Cy2 conjugated rabbit anti-mouse (1:200 in PBS) and Cy3 conjugated donkey

anti-rabbit (1:400 in PBS) for 1 hour at room temperature. After washing with PBS three times again, the sections were also counterstained with DAPI (diluted in PBS) to stain all nuclei for a better orientation. The slides were covered with mountain medium (Microscopy Aquatex) and cover glasses were added.

2.4.2. Occludin/Von Willebrand Faktor/IgG

For IgG staining, a different protocol was necessary. After drying the slides for 1 hour, they were placed in 100 % ethanol for 1 hour at 4°C and for 3 min in Acetone at −20°C. After washing with PBS they were blocked with 0.1% PBST (=PBS and Triton X-100, to enhance antibody penetration) with 5% NDS for 30 min. This pre-treatment provides also a better adhesion of the sections on the slides.

The sections were incubated in the wet chamber with the primary guinea pig anti-occludin (1:10 in 0.1% PBST with 5% NDS), rabbit anti-VWF (1:4000 in 0.1% PBST with 5% NDS) and Cy3 conjugated donkey anti-rat IgG (1:600 in 0.1% PBST with 5% NDS) over night at 4°C. After washing three times with PBS (5 minutes each), the sections were incubated for 1 hour at room temperature in the wet chamber with the second antibodies: AMCA conjugated donkey anti-guinea pig (1:30 in PBS), Cy2 conjugated donkey anti-rabbit (1:200 in PBS) and again Cy3 conjugated donkey anti-rat IgG (1:600 in PBS) for a better staining intensity.

After washing again with PBS three times for 5 min each, the slides were covered with mountain medium and cover glasses.

2.5. Analysis of the staining

To investigate occludin expression, the sections were analysed using a fluorescence microscope (OLYMPUS BX51) and pictures were taken with a colour fluorescence camera (OLYMPUS DP50). The occludin was counted in every spinal cord quadrant (ipsilateral ventral, ipsilateral dorsal, contralateral ventral, contralateral dorsal) and the expression within each vessel was classified as “intact” (clear lines of occludin), “partial” (less than 80% occludin per vessel), “foggy” (diffuse distribution of occludin) and “lacking”. Occludin was counted in relation to blood vessel stained by the anti-VWF-antibody. Between 60 and 100 vessels per quadrant were randomly counted with this method.

To analyse the IgG staining intensity as a marker for the BSCB integrity, we used a semi-quantitative method. Pictures of the ipsilateral dorsal region of the slides were taken from the fluorescence microscope (OLYMPUS BX51) using a colour fluorescence camera (OLYMPUS DP50). To subtract the background, a color threshold was set and the pictures where binarized with this threshold using CellD 3.3. (Olympus). Through the binarization, the images were converted in white pixels, which represent the IgG staining and black pixels (background). Afterwards, the integrated density of the whole images was measured using ImageJ software (from National Institute of Health). The integrated density equals the product of area and mean grey value (mean grey value = the sum of the gray values of all the pixels in the selection divided by the number of pixels). The images were taken blinded from the ipsilateral dorsal region, making an effort to include as many vessels as possible and avoiding the border to the white matter. To standardize the analysis, the slides from one staining procedure were analysed immediately with the same exposure time (200 ms for IgG and VWF staining), magnification (40x air lens resulting in 400x total magnification) and threshold settings. Every analysis included sections from vehicle animals.

To investigate a direct correlation between IgG extravasation and occludin distribution, the triple staining (2.4.2.) was analysed by counting IgG positive vessels and classifying the occludin integrity on these vessels. 72 hour post-carrageenan groups of the IgG experiment were used (n=7) and occludin distribution of vessels with IgG from the ipsilateral dorsal region was classified as in the occludin experiment. 15-40 vessels per animal were analysed.

Representative pictures of the triple staining were made with the black/white fluorescence camera OLYMPUS XM10 for a better resolution. To edit contrast and size of representative pictures showed in the figures, Photoshop CS4 was used.

2.7. Experimental design

The following groups of animals were used for the experiments:

Occludin distribution: males-lumbar region: vehicle (n=8), 3 hours (n=5), 24 hours (n=5), 72 hours (n=8), 5 days (n=5), subcutaneous (n=5). Females-lumbar region: vehicle (n=5), 3 hours (n=5), 24 hours (n=5), 72 hours (n=5).

IgG extravasation: males-lumbar region: vehicle (n=5), 24 hours (n=4), 72 hours (n=5), 5 days (n=4), subcutaneous (n=5). The thoracal region was also analysed in most animals.

Males-thoracal region: vehicle (n=4), 24 hours (n=4), 72 hours (n=4), 5 days (n=4), subcutaneous (n=4). Females-lumbar region: vehicle (n=5), 24 hours (n=4), 72 hours (n=5), 5 days (n=4), subcutaneous (n=4).

Animals from the 72 hour timepoint of the IgG experiment with the most prominent IgG extravasation were used for the analysis of the triple staining: males-lumbar region 72 hours (n=3), females-lumbar region 72 hours (n=4)

2.8. Statistics

Statistical analyses and plotting were performed using the software GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). One-way analysis of variance (ANOVA) followed by Dunnett's posthoc test was used to compare the semiquantitative occludin distribution from every timepoint group with the vehicle treated group within each quadrant for male and female rats separately. The same statistical analysis was also used to compare the integrated density of IgG in the ipsilateral dorsal quadrant to compare every timepoint with the vehicle group in male and female rats. Two-tailed unpaired t-test was used to compare between males and females timepoint groups of the integrated density of the IgG staining and male and female vehicle and 72 hours groups of intact occludin staining. One-way ANOVA followed by Bonferroni's Multiple Comparison test was used to compare the occludin classification of the vessels with IgG staining in the triple staining. *P*-values lower than 0.05 were considered significant. All data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Occludin distribution

In the first experiment, the effect of a peripheral carrageenan-induced inflammation on the expression of the tight junction protein occludin in the spinal cord was investigated. Male and female rats were used and the vascular occludin distribution was investigated at several timepoints after carrageenan-injection. Male rats displayed significantly decreased intact occludin at 72 hours post-injection in the ipsilateral dorsal (70.9% ± 2.5; $p<0.05$) and contralateral dorsal (68.2% ± 2.5; $p<0.01$) regions compared to vehicle treated animals (ipsilateral dorsal 82.5% ± 2.9; contralateral dorsal 81.4 ± 2.2) (**Figure 5; Figure 7**). There

were no significant differences at 72 hours post-injection in the ipsilateral ventral (70.9% ± 2.6) and contralateral ventral (70.6% ± 2.6) regions. There were no significant differences at 120 hours (5 day) post-injection as compared to control. Furthermore, the subcutaneous injection of the same dose of λ-carrageenan into the neck of male rats showed no significant difference compared to vehicle treated male rats.

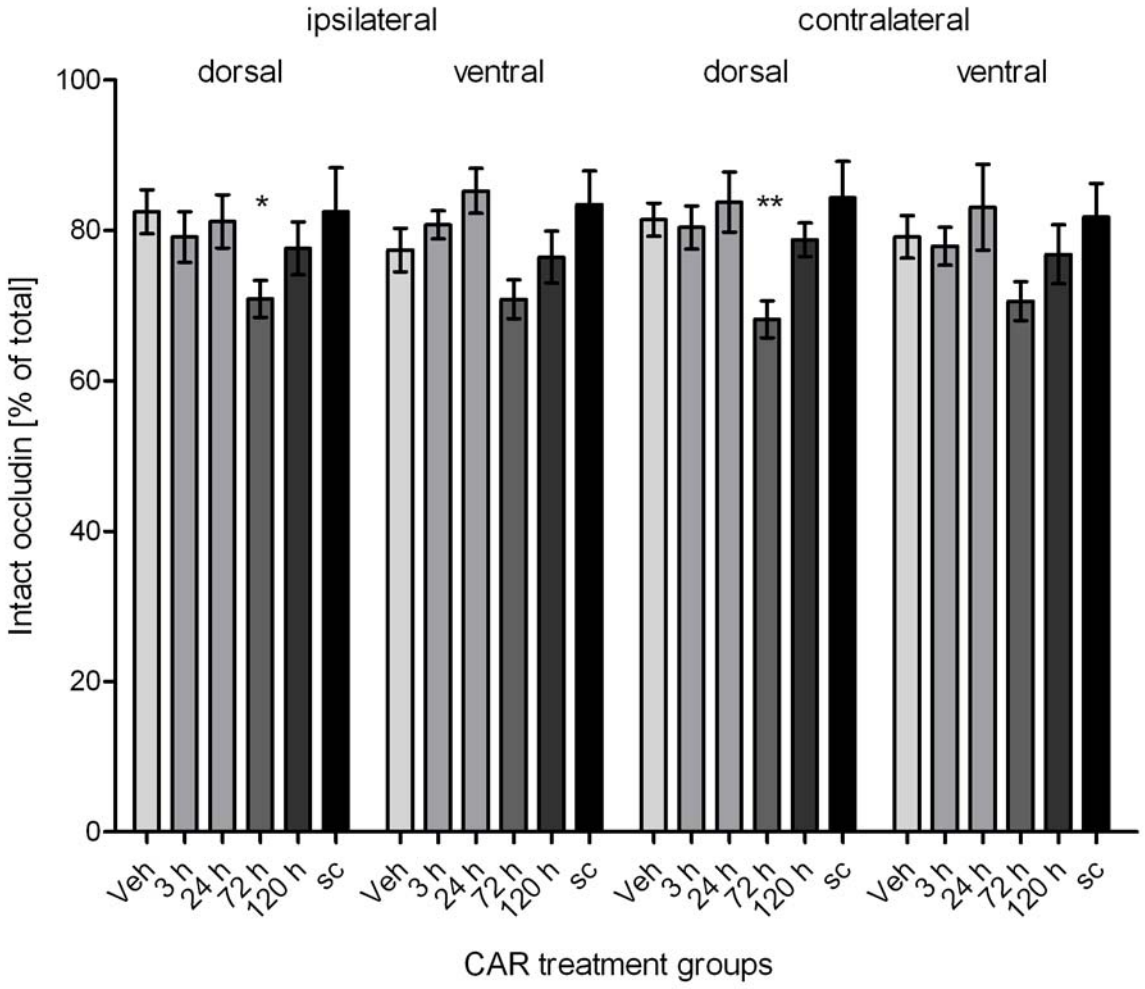

Figure 5: Effect of subplantar λ-carrageenan injection in male rats on the occludin expression in the spinal cord. Occludin distribution was analysed in comparison to the vessels within every spinal cord quadrant. As compared to control groups, occludin classified as intact was significantly decreased 72 hours after carrageenan in the ipsilateral dorsal and contralateral dorsal regions (CAR=carrageenan, Veh=vehicle, sc=subcutaneous injection, *$p<0.05$, **$p<0.01$; tested with one-way ANOVA followed by Dunnett's post hoc test). Veh: n=8, 3 h: n=5, 24 h: n=5, 72 h: n=8, 120 h: n=5, sc: n=5.

The occludin distribution in female rats showed no significant difference at 3, 24 or 72 hours post-carrageenan as compared to vehicle treated animals (**Figure 6**). Since we found no differences, we did not include a 5-day- and subcutaneous-female-group as with the males.

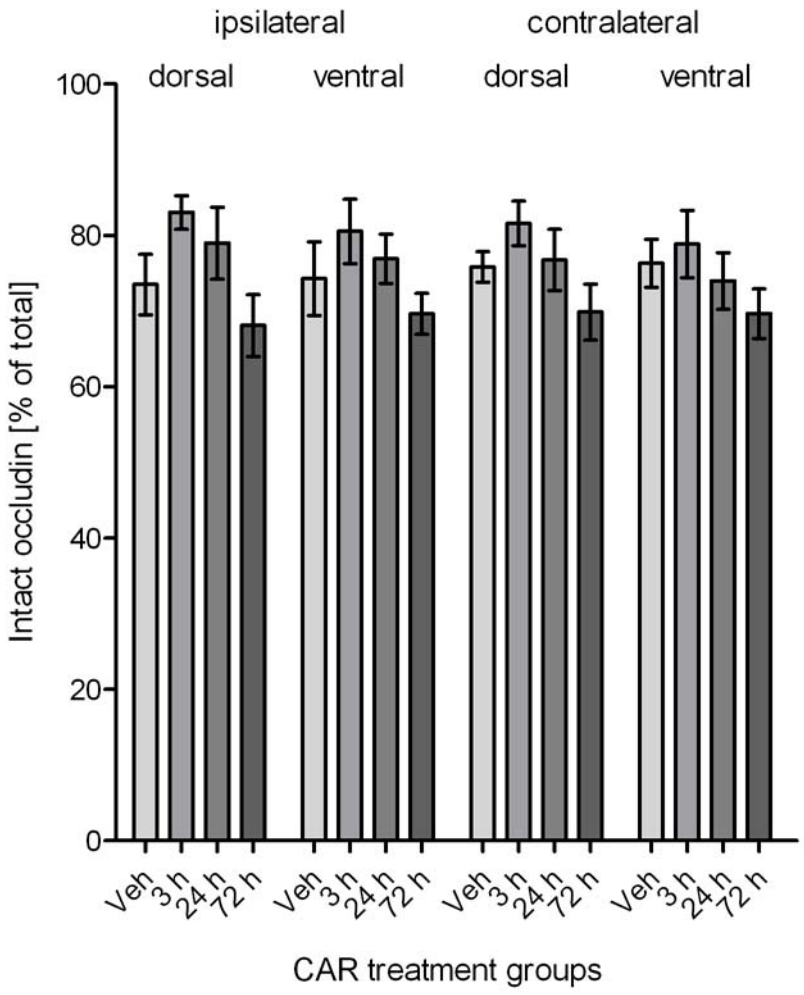

Figure 6: Intact occludin expression in the spinal cord of female rats after subplantar λ-carrageenan injection. Occludin distribution was analysed in comparison to the vessels within every spinal cord quadrant. One-way ANOVA followed by Dunnett's post hoc test showed no significant differences between the vehicle group and any timepoint (CAR=carrageenan, i=ipsilateral c=contralateral, Veh=vehicle). n=5 per group.

Within the vessels with abnormal occludin, most of them had what was classified as "partial" occludin, few had "foggy" occludin and a small percentage had no occludin at all (**Table 1**). Foggy occludin means a diffusive pattern of occludin which indicate a dislocation of the protein, probably due to damaged vessels and thus destroyed tight junctions. One-way ANOVA followed by Dunnett's posthoc test showed a significant increase of partial occludin after 72 hours post-carrageenan in males in the contralateral dorsal side (24.5% ± 1.7) and lost occludin in the ipsilateral dorsal side (4.6% ± 1.2). But those values are in the range of the data of the other quadrants in the same group. Foggy and completely lost occludin

appeared infrequently. Partial occludin had a characteristic punctuate distribution and was clearly distinguished from the clear lines of the intact occludin distribution (arrows in **Figure 7**).

Table 1: Semiquantitative classification of the occludin distribution after several timepoints of subplantar λ-carrageenan injection. Data show % of total counted vessels ± SEM (d=dorsal, v=ventral, Veh=vehicle, sc=subcutaneous injection, *p<0.05, **p<0.01, p***<0.001; tested with one-way ANOVA followed by Dunnett's post hoc test).

	Veh male	3 hrs male	24 hrs male	72 hrs male	5 days male	72 hrs male sc	**Veh fem**	3 hrs fem	24 hrs fem	72 hrs fem
N	8	5	5	8	5	5	5	5	5	5
Intact										
Ipsi d	82.5% ± 2.9	79.1% ± 3.4	81.2% ± 3.5	70.9% ± 2.5*	77.6% ± 3.5	82.5% ± 5.9	73.5% ± 4.0	83.0% ± 2.2	79.0% ± 4.8	68.1% ± 4.1
Ipsi v	77.4% ± 2.9	80.8% ± 1.9	85.3% ± 3.0	70.9% ± 2.6	76.5% ± 3.5	83.5% ± 4.4	74.3% ± 4.9	80.5% ± 4.3	76.9% ± 3.3	69.6% ± 2.7
Contra d	81.4% ± 2.2	80.4% ± 2.9	83.8% ± 4.0	68.2% ± 2.5**	78.8% ± 2.2	84.4% ± 4.8	75.8% ± 2.0	81.6% ± 2.9	76.8% ± 4.0	69.9% ± 3.7
Contra v	79.2% ± 2.8	77.9% ± 2.5	83.1% ± 5.7	70.6% ± 2.6	76.8% ± 3.9	81.8% ± 4.4	76.3% ± 3.2	78.8% ± 4.4	74.0% ± 3.8	69.7% ± 3.3
Partial										
Ipsi d	12.2% ± 2.4	13.1% ± 2.0	7.5% ± 2.8	20.9% ± 2.2	19.4% ± 4.5	15.0% ± 4.4	17.9% ± 2.7	14.1% ± 2.1	15.2% ± 4.8	22.1% ± 4.7
Ipsi v	15.6% ± 3.7	13.1% ± 2.0	9.6% ± 3.6	24.2% ± 2.1	22.3% ± 3.1	13.4% ± 4.0	17.5% ± 4.6	14.6% ± 3.9	16.7% ± 2.6	21.9% ± 2.5
Contra d	10.7% ± 2.0	16.0% ± 2.0	10.3% ± 3.8	24.5% ± 1.7***	17.7% ± 2.8	12.3% ± 4.4	18.3% ± 2.2	13.7% ± 2.3	16.1% ± 3.4	22.2% ± 4.8
Contra v	16.1% ± 2.6	17.2% ± 3.5	12.0% ± 4.9	21.2% ± 1.3	21.5% ± 3.6	16.3% ± 3.8	18.8% ± 2.4	15.4% ± 3.2	18.5% ± 3.3	22.6% ± 3.3
Foggy										
Ipsi d	4.5% ± 1.8	4.6% ± 1.8	9.6% ± 2.0	3.6% ± 1.5	0.6% ± 0.6	1.0% ± 0.6	6.4% ± 3.5	1.8% ± 1.5	5.5% ± 2.9	8.0% ± 3.1
Ipsi v	6.1% ± 1.9	4.0% ± 1.7	4.9% ± 1.3	3.1% ± 1.5	0.6% ± 0.6	1.9% ± 1.2	4.5% ± 2.7	2.3% ± 1.4	5.3% ± 2.4	6.9% ± 2.9
Contra d	6.0% ± 1.8	0.7% ± 0.5	5.4% ± 1.4	4.4% ± 1.6	0.2% ± 0.2	1.7% ± 1.2	5.1% ± 2.4	3.0% ± 1.3	4.7% ± 1.4	7.4% ± 3.2
Contra v	4.4% ± 1.6	3.0% ± 0.5	4.3% ± 1.7	6.5% ± 2.2	1.6% ± 1.0	0.8% ± 0.5	2.6% ± 0.5	2.3% ± 0.9	5.5% ± 2.1	6.6% ± 3.9
No										
Ipsi d	0.8% ± 0.5	3.2% ± 1.0	1.7% ± 1.2	4.6% ± 1.2*	2.3% ± 1.3	1.5% ± 1.0	2.2% ± 1.5	1.0% ± 0.5	0.3% ± 0.3	1.9% ± 0.7
Ipsi v	0.9% ± 0.4	2.2% ± 0.9	0.3% ± 0.3	1.9% ± 0.6	0.6% ± 0.6	1.3% ± 0.8	3.8% ± 1.4	2.6% ± 1.3	1.1% ± 0.6	1.7% ± 0.7
Contra d	1.9% ± 0.4	2.8% ± 0.9	0.6% ± 0.6	3.1% ± 0.7	3.3% ± 1.1	1.6% ± 0.2	0.8% ± 0.8	1.7% ± 0.8	2.5% ± 1.3	0.5% ± 0.3
Contra v	0.4% ± 0.2	1.9% ± 0.7	0.6% ± 0.6	1.8% ± 0.7	0.0% ± 0.0	0.8% ± 0.8	2.3% ± 0.8	3.4% ± 1.8	2.1% ± 0.8	1.1% ± 0.5

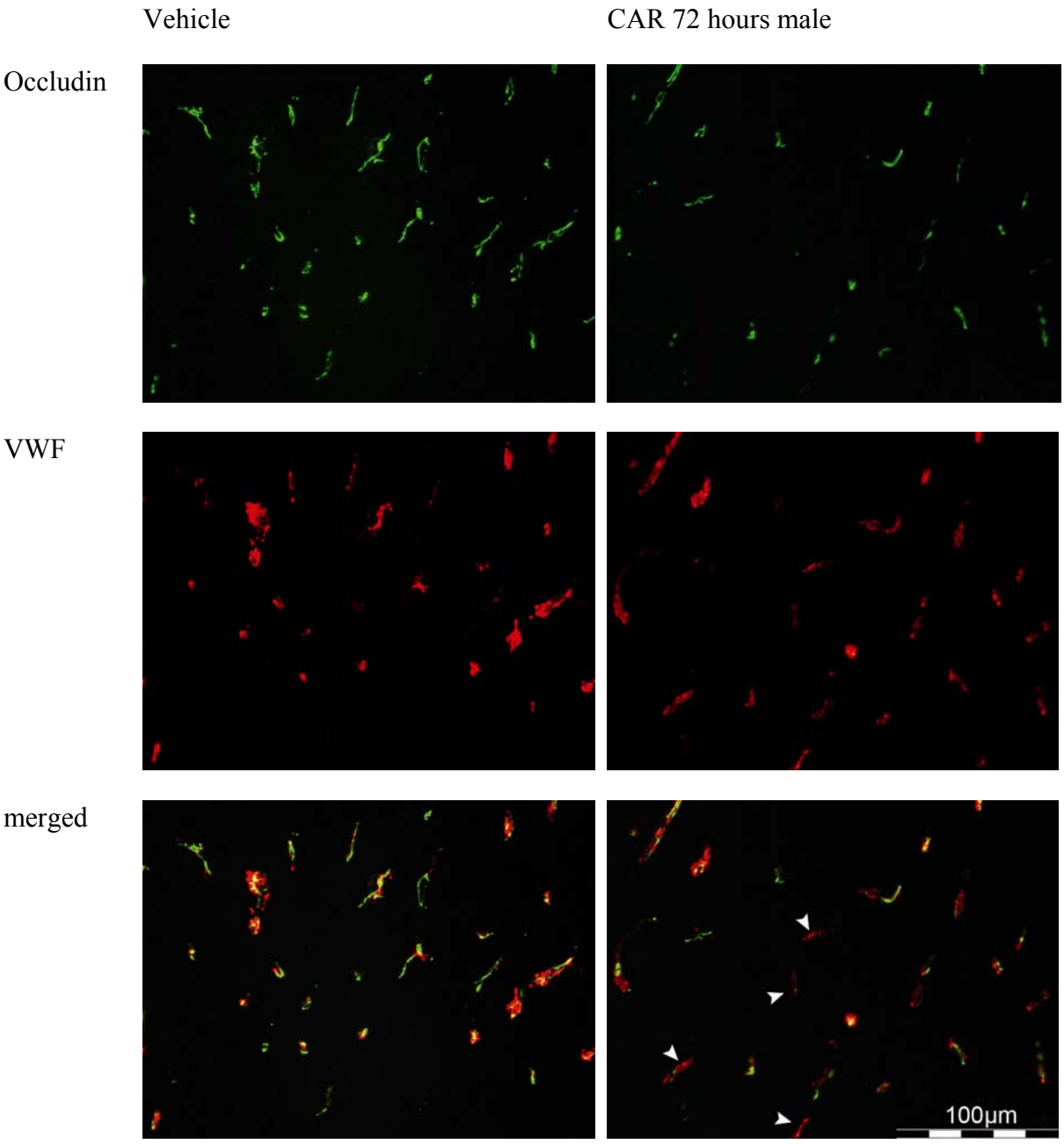

Figure 7: Immunohistochemical analysis of the tight junction protein occludin 72 hours after subplantar λ-carrageenan (CAR) injection (right) compared to vehicle treatment (left) in male rats. Occludin is stained with anti-occludin antibody (second antibody conjugated with Cy2, green). Vessels are co-stained with anti-von Willebrand Factor (VWF) (second antibody conjugated with Cy3, red). Yellow colour shows overlapped staining. Arrows reveal abnormal occludin distribution.

3.2. IgG extravasation

To investigate the permeability of the BSCB to large molecules after carrageenan-injection, IgG, which is normally not found in the parenchyma of the CNS, was analysed in perfused animals by immunohistochemistry and measurement of the integrated density of the fluorescence staining. The time course after carrageenan-injection was investigated with male and female groups for 24 hours, 72 hours and 5 days (120 hours). Furthermore, a group with subcutaneous injection into the neck was used to analyse a possible systemic effect of the carrageenan-injection on the IgG extravasation.

The integrated density in the lumbar region was significantly increased ($p<0.001$, tested with one-way ANOVA followed by Dunnett's post hoc test) 72 hours post-injection in male (57.1 ± 26.5) and female rats (108.9 ± 42.3) as compared to vehicle groups (female vehicle: 16.0 ± 9.2; male vehicle: 3.9 ± 1.3) (**Figure 8A**, **Figure 9A**) and furthermore in the thoracal ispilateral dorsal region of males (27.3 ± 13.8) compared to vehicle group (4.3 ± 2.5). Unpaired, two-tailed t-test showed a significant difference of the integrated density after 72 hours between males and females ($P=0.048$). There were no significant changes at any other timepoint or in the subcutaneous group.

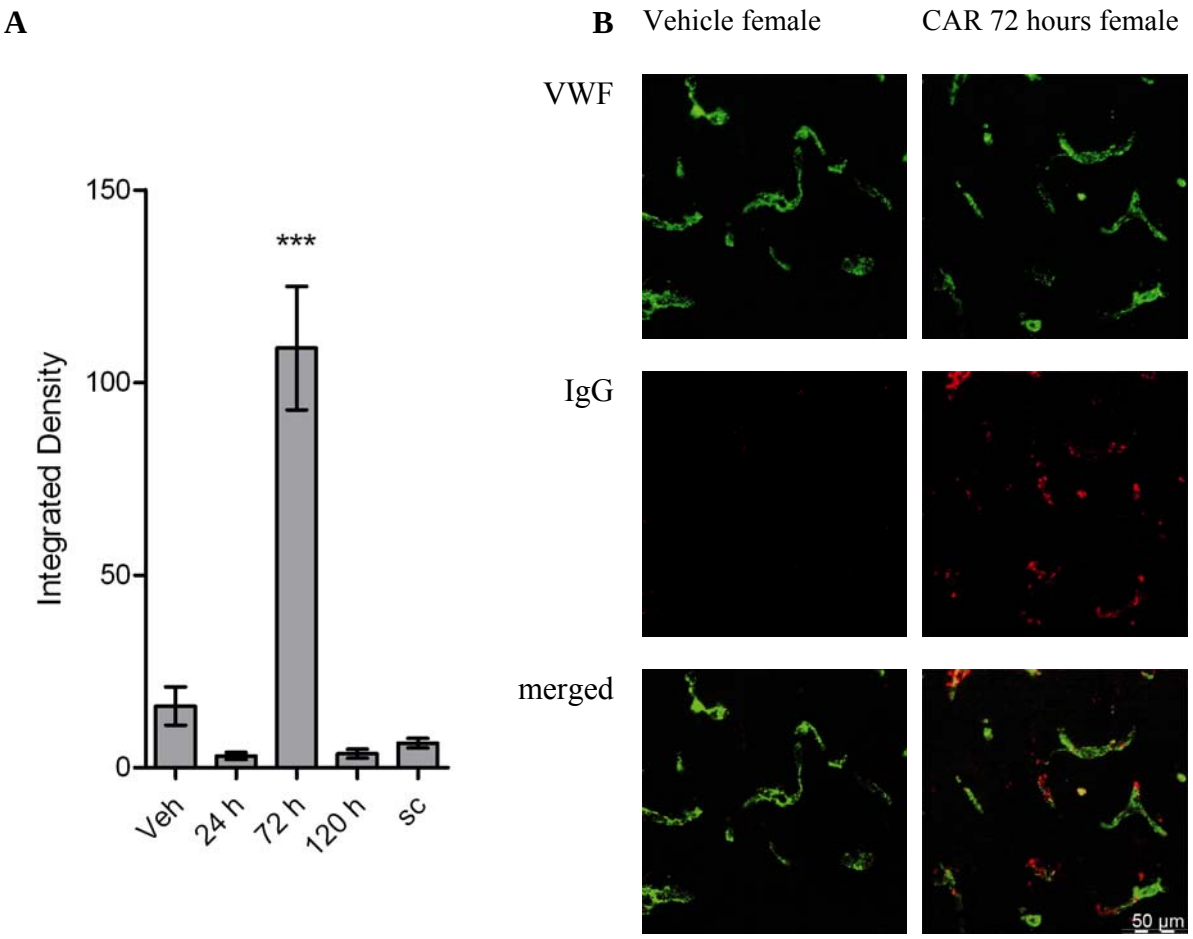

Figure 8: IgG extravasation in the lumbar region of female rats (n=4-5). **A**: Semiquantitative analysis of the immunohistochemical IgG staining in the spinal cord after several timepoints after subplantar λ-carrageenan (CAR) injection. The integrated density of IgG is significantly increased (***$p<0.001$; one-way ANOVA followed by Dunnett's post hoc test) 72 hours post-injection compared to the vehicle treated group. **B**: Fluorescence microscopy shows a clear difference in IgG staining between vehicle treated females and 72 hours after carrageenan injection. Von Willebrand Factor (VWF) was used to show the endothelial cells of the blood-spinal cord vessels (second antibody conjugated with Cy2, green). IgG (second antibody conjugated with Cy3) diffuse outside of the vessels into the spinal parenchyma (merged pictures).

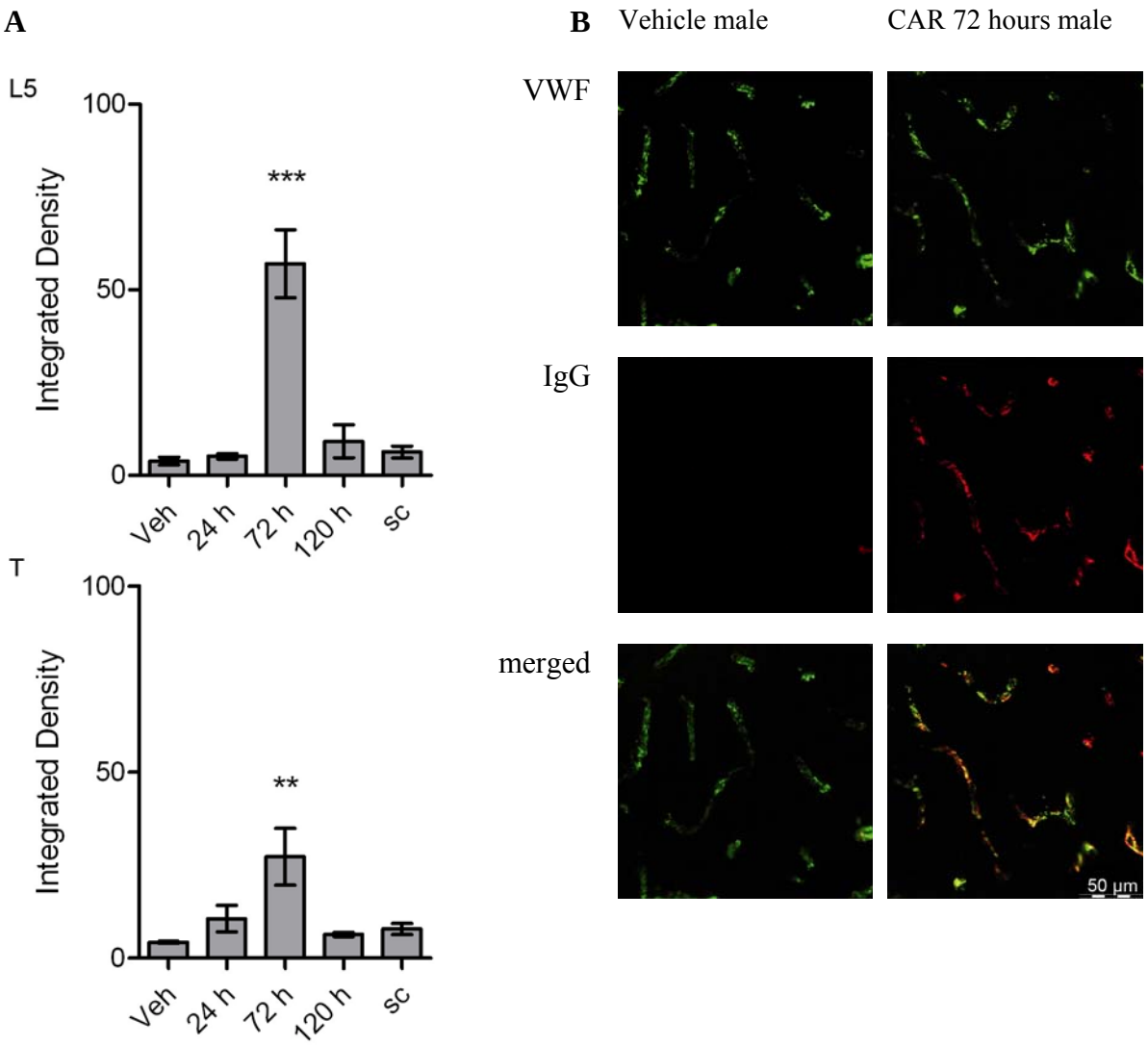

Figure 9: IgG extravasation in the lumbar (**L5**, n=4-5) and thoracal (**T**, n=4) region of male rats. **A**: Semiquantitative analysis and calculation of the integrated density of IgG staining in the spinal cord after several timepoints of subplantar λ-carrageenan (CAR) injection. There is a significant increase of the integrated density of IgG 72 hours post-injection in the ipsilateral dorsal lumbar region (***$p<0.001$; one-way ANOVA followed by Dunnett's post hoc test) and the thoracal region (**$p<0.01$) compared to the vehicle treated group. **B**: Representative pictures of the fluorescence microscope. Von Willebrand Factor (VWF) was used to show the endothelial cells of the blood-spinal cord vessels (second antibody conjugated with Cy2, green). IgG antibody was conjugated with Cy3 (red).

3.3. Correlation between IgG extravasation and occludin distribution

In a further experiment we analysed the occludin distribution in vessels with IgG staining. For this investigation a triple staining of von Willebrand Faktor, IgG and occludin was

performed (**Figure 10B**). If occludin has a direct effect on the paracellular permeability, we assumed, that vessels with IgG extravasation shows abnormal occludin staining. However, 80.7% ± 2.0 of the counted vessels with IgG had intact occludin staining (**Figure 10A**).

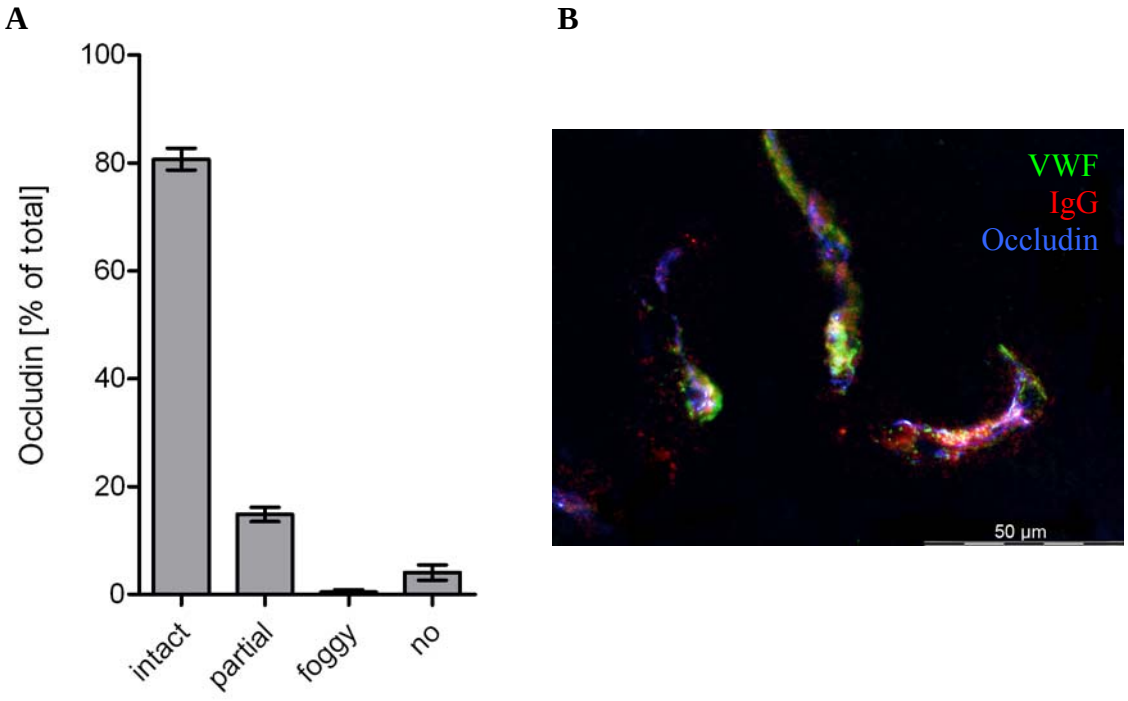

Figure 10: **A**: Correlation analysis between vessels with IgG extravasation and occludin distribution. Most of the vessels with IgG had intact occludin staining compared to the other classifications ($p<0.0001$, $n=7$; tested with one-way ANOVA followed by Bonferroni's Multiple Comparison test). **B**: Triple-staining of VWF (second antibody conjugated with Cy2, green), Immunoglobulin G (IgG, antibody conjugated with Cy3, red) and occludin (second antibody conjugated with AMCA, blue). Yellow and white show an overlapping of the staining.

4. Discussion

In the current study, alterations in the BSCB due to peripheral λ-carrageenan induced inflammation were investigated. Changes in the distribution of the tight junction protein occludin and altered BSCB permeability to immunoglobulin G were analysed in male and female rats. After 72 hours of carrageenan injection, there was a significant decrease of normal occludin in ipsilateral and contralateral dorsal side in males. Thus, the changes in occludin expression were not restricted only to the ipsilateral side since the peripheral

inflammation had a more widespread effect in the spinal cord. The innervation of primary afferent fibres from the hindpaw may explain the greater effect on the ipsilateral lumbar dorsal side. However, this is unlikely considering the trend of the ipsilateral and contralateral ventral side and the values which are all in the same range after 72 hours in males. The findings of Beggs and colleagues (2010) also showed altered BSCB properties throughout the whole spinal-cord after peripheral nerve injury, sciatic nerve constriction and C-fibre stimulation and there was no limitation to any quadrant. Interestingly, the peripheral inflammation had no significant effect on the occludin distribution in female rats. In previous studies, female rats had a lower nociceptive threshold than males after carrageenan-induced inflammation (Tall and Crisp 2004) and a higher increase of the BBB permeability after epileptic seizures as compared to male rats (Oztas et al. 1992). The absent effect in females could due to the lower base level of intact occludin in vehicle treated females compared to vehicle treated males. Two-tailed unpaired t-test analysis of occludin in all four quadrants combined showed a significant difference between vehicle treated females as compared to vehicle treated males, but not between females and males 72 hours after injection.

Compared to other analysis methods of tight junction proteins like Western blot, the analysis method for occludin used in this study has some advantages and disadvantages. The major advantages are the distinction between the ipsi-/contralateral and dorsal/ventral quadrant and the visualization of the occludin distribution pattern in the microvessels of the spinal cord. A bias in this semiquantitative analysis of counting and classification of occludin distribution is reduced because of the randomized and blinded study design. This method has, however, also some limitations. It is not possible to distinguish between the different phosphorylation states of occludin – which may be directly involved in its assembly – as is the case with Western blot analysis (Sakakibara et al. 1997). The foggy occludin pattern may indicate a dislocation of occludin from the vessels into the parenchyma. This may happen due to damaged vessels or it could be a staining artefact. Furthermore, it is not clear if lost occludin staining really means a total degradation of this protein or loss due to the tissue sectioning. This could also occur in the partial classification but is unlikely because of the typical punctuate distribution also characterized in another study (Morgan et al. 2007). Since, these errors occurred in all groups and the groups were compared with the vehicle group, it is likely that they have no influence on the relative values and significant P-values in our study.

In the further progress of this study, we wanted to investigate the effect of the peripheral inflammation on the permeability of the BSCB. Again, male and female rats were used. The endogenous immunoglobulin G (IgG) which normally does not cross the BBB was used as a

marker for changes of the paracellular permeability. The integrated density of the IgG staining in the ipsilateral dorsal lumbar region was significantly increased 72 hours after carrageenan-injection in both male and female rats and also in the thoracal region of males. These results match with the results of the decreased occludin distribution in males. This could be an indicator that degraded occludin in the tight junctions leads to the opening of the BSCB and which is consistent with other studies showed occludin as a determinant of BBB permeability (Hirase et al. 1997; Chen et al. 1997). However, it is more likely that the occludin degradation is only an additional effect of the inflammation and not directly responsible for decreased BSCB permeability, because there is also a large increase of IgG extravasation in females, but no significant difference in normal occludin compared to vehicle. This effect is actually larger in females than in males ($p=0.048$), what is consistent with the study of Oztas and colleagues (1992) showing an increased permeability in females compared to males after epileptic seizures. Increased BBB permeability is often associated with a decreased occludin distribution, especially in animal models with peripheral inflammation (Huber et al. 2001; Huber et al. 2002; Brooks et al. 2005; Brooks et al. 2006). However, in some cases it has been shown that increased permeability of the BBB can occur without changes in the expression of occludin (Hirase et al. 1997).

The direct comparison between IgG extravasation and abnormal occludin showed no direct correlation. Most of the vessels which had IgG staining exhibited normal occludin. This direct correlation – which has never been reported before – was possible with a triple staining of von Willebrand Faktor, IgG and occludin. Our results suggest that opening of the BSCB is possible without a disturbance in occludin protein. Using a long perfusion of the animals with PB, the entire blood was flushed out making it likely that the IgG staining should only show the IgG efflux into the parenchyma of the spinal cord, as shown in previous studies (Pelegrí et al. 2007; Chen et al. 2008). However, some vessels seemed to exhibit a colocalization of IgG staining and von Willebrand Faktor. A cross-staining between the antibodies can be excluded due to many control stainings and by the fact that nearly no IgG staining was seen in the vehicle treated groups. IgG may bind on the surface of the endothelial cells or get transported inside the cells. For example, specialized Fc-receptors can bind IgG but they are usually expressed on the surface of immune cells and not on endothelial cells. Only one Fc-receptor which binds IgG was found on the endothelial cells of neonatal but not in adult rodents (Borvak et al. 1998). Furthermore, IgG is not able to diffuse into endothelial cells. One plausible explanation of this event could be that IgG was able to enter into the endothelial cells when the lipid walls of the cells are destroyed. But irreversible damaged

endothelial cells can not explain this occurrence only after 72 hours post-injection and return of the basal conditions after 5 days. A precise investigation of the colocalization with a colocalization analysis was not possible due to the low signal-noise-ratio.

Analysis of IgG extravasation and calculation of the integrated density was performed in a blinded manner and randomized, thereby reducing bias. However, it is a semiquantitative analysis because the taken pictures contained a different number of vessels. Thus, the relative values and significances are representative but not the total values.

The subcutaneous injection of λ-carrageenan into the back of the rats showed no changes in the occludin distribution and IgG extravasation in the spinal cord. Thus, λ-carrageenan has likely no systemic effect but provokes a local peripheral inflammation. The underlying mechanisms of the peripheral λ-carrageenan inflammations leading to a break down of the BSCB and degradation of IgG are still unknown. Due to the delayed effect it is probable that the peripheral inflammation causes the local release of mediators which triggers a cascade of events. Transcription of genes and translation of proteins may be involved in this process that finally leads to structural changes of the BSCB. Since carrageenan leads to an inflammatory pain, activation of nociceptive neurons may be involved in this cascade of events. It is known that the carrageenan-inflammation activates different pathways of both the nervous and the immune system (Di Rosa et al. 1971). Campos et al. showed that nociceptive signalling is one important mechanisms in the peripheral λ-carrageenan inflammation by blocking the changes in [^{14}C]sucrose permeability and occludin expression in the BBB with an local anaesthetic drug (Campos et al. 2008). This local anaesthetic drug inhibits the afferent nociceptive signalling to the spinal cord and may prevent the mediator release, leading to alterations in the BBB. Another study used the same inflammatory animal model and blocked the effect on the BBB by inhibiting the production of prostaglandins in the brain (Brooks et al. 2008). Thus, it is obvious that different mechanisms are playing a role in this inflammatory model and they can lead to an immediate or delayed effect, like in our study. Previous studies of chronic constriction injury and spared nerve injury showed no immediate effects on the BSCB, but an increased permeability to Evans Blue after 24 and 72 hours (Beggs et al. 2010). Furthermore, peripheral inflammation due to injection of CFA (Complete Freud's Adjuvant) showed also a delayed effect on the permeability and tight junction assembling in the BBB 72 hours post-injection (Huber et al. 2001; Brooks et al. 2005; Brooks et al. 2006). There are a few studies shown the effect of carrageenan-induced peripheral inflammation on the permeability and tight junction proteins in the BBB over a longer time period. One research group investigated this effect after several timepoints after subplantar λ-

carrageenan injection in rats (Huber et al. 2002; Hau et al. 2004). They found biphasic changes of increased permeability to [^{14}C]sucrose and a decreased occludin distribution (shown by Western blot) with an early effect after 1 to 6 hours and a delayed effect after 48 hours and a return to base levels between this timepoints. We did not see an early effect in the 3 hour timepoint group and thus, did not investigate the IgG staining at the same timepoint. Our different results to the study shown a biphasic effect in the BBB could be due to different methods used for measuring the barrier permeability and occludin distribution. Furthermore, other findings affirm that there are differences in the permeability between spinal cord and brain with an increased permeability of the spinal cord (Pan et al. 1997a; Pan et al. 1997b). This may explain our clear significant IgG extravasation compared to the vehicle group. Further investigations of permeability changes to IgG at earlier timepoints after carrageenan-injection may discover an additional acute effect. Also additional methods with Western blot analysis of occludin and the usage of other permeability tracers could affirm our results. The investigation of the underlying mechanisms of sex differences in the BSCB properties would also be an important approach for further studies. Analysis of circulating sex hormones and oestrus cycle stages of the female rats could be performed.

In conclusion, this study extends our knowledge about properties of the spinal cord during peripheral inflammatory pain. This may have clinical implications for treatment of peripheral inflammation. It is important to know that pain conditions in the periphery next to many other diseases provoke a breakdown of the protective barrier between the blood circulation and the central nervous system. The consequences may be an increased efflux of harmful substances out of the blood into the parenchyma of the spinal cord that can lead to an irreversible damage of neurons. Other effects may be important for drug therapy because drugs which normally do not cross the BSCB and BBB can have more side effects due to their impact on the nervous system. On the other hand, the specific opening of the BBB and BSCB is one important approach for pharmacological therapies addressing central nervous system diseases. The investigation of the BBB and BSCB offers an important field of research and can help us gain knowledge for the development of drug-delivery systems to the brain.

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Abstract

The protective barrier between the blood and the central nervous system is characterized by a complex interaction between endothelial cells forming the microvessels and different cell types of the nervous system. They provide a microvascular unit and are essential for nutrient supply and the "fence" function of the barrier. Tight junctions between the endothelial cells are responsible for restriction of the paracellular diffusion pathway. Many pathological conditions including chronic pain may have a major impact on the integrity of the BBB and BSCB. It has been shown that peripheral inflammations can increase the permeability of the BBB and change the tight junction protein assembly. Some differences are already known between the BBB and the BSCB (e.g. the higher permeability of the BSCB), although most previous studies focused on the BBB during pathological conditions. The clinical relevance of the BSCB may be important in relation to pain mechanism. In this study we investigated the effect of a peripheral inflammation in rats on tight junction assembling and the permeability of the BSCB by semiquantitative analysis methods. We also investigated sex differences. The results show a significant decrease of normal occludin distributions in the dorsal lumbar region of males 72 hours after λ-carrageenan induced inflammation but no differences in females. The extravasation of immunoglobulin G (IgG) into the spinal cord parenchyma was significantly increased 72 hours post-injection in the ipsilateral dorsal lumbar region of males and females with a higher increase in females and a further increase in the thoracal region of males. Direct comparison of IgG extravasation and occludin distribution indicated no correlation between increased BSCB permeability and abnormal occludin.

These findings showed a large but delayed impact of a peripheral λ-carrageenan inflammation on the properties of the BSCB which may have clinical implications. This inflammatory animal model is known to evoke robust hyperalgesia and it is possible that this persistent activation of nociceptive neurons which innervate the spinal cord release mediators and trigger a cascade of events that finally leads to a reversible degradation of tight junction proteins and increased permeability in the BSCB. Our study have clinical implications for the treatment of inflammatory pain and shows the impact of peripheral inflammations – which occur during different diseases – on the BSCB. This barrier break down can lead to an influx of pathogens into the central nervous system and should be also considering during therapeutic drug administration which may lead to larger side effects due to an increased delivery.

Zusammenfassung

Die schützende Barriere zwischen Blut und dem Zentralen Nervensystem ist durch eine komplexe Wechselbeziehung zwischen den Endothelzellen der Blutgefäße und verschiedenen Zellen des Nervensystems charakterisiert und ist für die Aufrechterhaltung der Milieubedingungen im zentralen Nervensystem essentiell. Sogenannte Tight Junctions zwischen den Endothelzellen bilden eine hochselektive parazelluläre Diffusionsbarriere. Verschiedene Krankheiten, wie auch der chronische Schmerz, können jedoch die Integrität der Blut-Hirn- und Blut-Rückenmarks-Schranke stark beeinflussen. Es ist bekannt, dass periphere Entzündungen die Permeabilität der Blut-Hirn-Schranke erhöhen und die Anordnung der Proteine in den Tight Junctions verändern können. Zwar sind einige Unterschiede zwischen der Blut-Hirn- und Blut-Rückenmarks-Schranke bekannt (wie zum Beispiel eine höhere Permeabilität der Blut-Rückenmarks-Schranke), die meisten bisherigen Studien konzentrieren sich jedoch hauptsächliche auf die Blut-Hirn-Schranke unter pathologischen Zuständen. Die Blut-Rückenmarks-Schranke könnte in Hinsicht auf Schmerzmechanismen von wichtiger klinischer Relevanz sein. In dieser Studie wurde die Auswirkung einer peripheren Entzündung in Ratten auf die Anordnung des Tight Junction Proteins Occludin und die Permeabilität der Blut-Rückenmarks-Schranke mit semiquantitativen Analysemethoden untersucht. In diesem Bezug wurden weiters Geschlechtsunterschiede erforscht. Die Ergebnisse zeigen eine signifikante Abnahme der intakten Verteilung von Occludin in der dorsal lumbalen Region der Männchen 72 Stunden nach der λ-Carrageen induzierten Entzündung. Bei Weibchen wurden keine Unterschiede gefunden. Der Austritt von Immunoglobulin G (IgG) aus dem Blut in das Rückenmarkparenchym war 72 Stunden nach der Carrageen-Injection in der ipsilateral dorsal lumbalen Region in Männchen und Weibchen signifikant erhöht, wobei dieser Effekt bei Weibchen höher war. Bei der Untersuchung der thorakalen Region in Männchen wurde auch dort ein erhöhter IgG-Austritt festgestellt. Der direkte Vergleich zwischen IgG-Austritt und Verteilung von Occludin wies auf keinen Zusammenhang zwischen erhöhter Permeabilität der Blut-Rückenmarks-Schranke und abnormaler Verteilung von Occludin hin.

Diese Ergebnisse zeigten eine starke, jedoch verzögerte Auswirkung einer peripheren λ-Carragen-Entzündung auf die Integrität der Blut-Rückenmarks-Schranke was zu klinischen Konsequenzen führen kann. Es ist bekannt, dass dieses Tiermodell für Entzündungen auch eine starke Hyperalgesie auslöst. Somit führt die andauernde Aktivierung von nozizeptiven Neuronen, welche das Rückenmark innervieren, vermutlich zur Ausschüttung von Botenstoffen, welche eine Kaskade von Ereignissen auslöst, die letztendlich einen reversiblen

Abbau von Tight Junction Proteinen und eine erhöhte Permeabilität der Blut-Rückenmarks-Schranke verursacht.

Diese Studie ist von klinischer Bedeutung für das Verständnis von Entzündungsschmerzen und zeigt die Auswirkungen von peripheren Entzündungen – welche während verschiedenen Krankheiten auftreten können – auf die Blut-Rückenmarks-Schranke. Durch die erhöhte Permeabilität dieser Barriere können Pathogene in das Zentralnervensystem gelangen, aber auch Medikamente, die somit zu stärkeren Nebenwirkungen führen könnten.

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