HEAD TRAUMA RELEASE OF HISTAMINE FROM DURAL MAST CELLS ALTERS BLOOD-BRAIN BARRIER: ATTENUATION WITH ZOLANTIDINE

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This study employed a new model of mild-to-moderate head trauma to specifically identify the role of dural mast cell (MC) histamine in trauma-induced increased permeability in the blood-brain barrier (BBB). A single line was scored partially through the left dorsal parietal skull. Immediately following the trauma, degranulation was seen in 39% of the MCs on the left and in 2% on the right. After a 20 min survival period, left duras showed 55% with MC degranulation (fewer with complete degranulation) compared to 34% on the right. In the other experiments two parallel lines were scored following the injection of Evan's blue. Histamine assay showed histamine increased in the left cortex to 154% at 5 min, 174% at 10 min, and 151% at 20 min. Fluorescent quantitation of extravasated Evan's blue at 20 min following the trauma gave an increase of 1385% over the value measured for the right cortex. Zolantidine, a selective histamine H_2 receptor antagonist, administered at 10- and 20- mg/kg 30 min before the trauma blocked 65% of the Evan's blue extravasation compared with the control and 2.5 mg group.

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CHAPTER 1

INTRODUCTION

Ten years ago traumatic brain injury (TBI) was referred to as a "silent epidemic"(Goldstein 1990). The annual incidence of TBI in the United States exceeds that for multiple sclerosis (Baun and Rothschild 1981), Alzheimer's disease (Brookmeyer, et al. 1998), and Parkinson's disease (Mayeux, et al. 1995) combined. The one million new cases of TBI in the United States each year include more than 50,000 deaths, and 70,000 to 90,000 people who develop serious life-long disabilities (Thurman and Guerrero 1999). More than 300,000 cases of TBI each year are sports or recreation related (Kelly 1999) as mild TBI (MTBI), also called concussions. At one time, the term concussion implied a head injury associated with loss of consciousness. Concussion has now been re-defined as a "trauma-induced alteration in mental status that may or may not involve loss of consciousness" (Report of the Quality Standards Subcommittee 1997). It has become of increasing concern that such "mild" injuries, or concussions, are not as benign as they formerly regarded (Tellier, et al. 1999, Voller, et al. 1999). MTBI not associated with loss of consciousness can alter mental and cognitive functioning for at least up to 7 days (Barth, et al. 1989, Collins, et al. 1999, Witol and Webbe 1994). Additional MTBIs further compound the initial injury, typically taking longer to resolve and with more evidence of long term deficits. The presence of a learning disability further increases the morbidity of the MTBI (Collins, et al. 1999). Most MTBIs each year are sports related [Ropper, 1998 #586; Collins, 1999 #561; Kelly, 1999 #543;

Matser,1998 #550; Matser, 1999 #549; Baroff, 1998 #551; Powell, 1999 #558]. Multiple MTBIs are now recognized as likely to confer some degree of permanent neurocognitive impairment (Cantu and Voy 1995) (Barth, et al. 1989, Collins, et al. 1999, Gronwall and Wrightson 1975, Matser, et al. 1999, Matser, et al. 1998, Witol and Webbe 1994).

A person suffering an MTBI, at best, is given a standard battery of neuropsycholgical tests (Collins, et al. 1999) and diagnostic x-rays and scans. Unless there is evidence of hemorrhage inside the skull requiring continued observation the person is sent home to recover without further medical attention. Many cases do not receive any medical evaluation. Even individuals who appear to overtly recover well experience perceivable difficulties not before present. These problems are often not seriously regarded by physicians who usually do not offer the patient any additional evaluation or rehabilitative care. The physician is often skeptical of causative association between the patient's complaints and the MTBI. This is not surprising since even recently published medical textbooks contain misinformation on MTBIs (Ropper 1998).

Numerous therapies to treat traumatic injuries of the brain and spine have shown promise in experimental animal models and some are in human clinical trials. There is presently, however, no drug therapy available that is significantly neuroprotective (Wahl and Stutzmann 1999).

In MTBI, as well as TBI, there are shared pathological factors. These include diffuse axonal injury and breakage (Povlishock, et al. 1983) and damage to the neuronal cytoskeleton (Saatman, et al. 1998). Despite progress at the research level, axonal

breakage is for all practical considerations a permanent damage event. Damage to neuronal cytoskeleton disrupts the normal signal receiving, processing, and sending functions of the neuron as well as normal housekeeping functions dependent upon intact neurofilament and microtubule systems. This renders affected neurons into a state of "metabolic shock" (Sutton, et al. 1994). The traumatized area is susceptible to localized swelling (edema formation) which further compromises the normal functional ability of neurons and glial cells. Elevated levels of excitotoxic transmitters, primarily glutamate, influx of calcium into neurons and glial cells, and high extracellular potassium levels are major problems (Katayama, et al. 1990, Ransom 1992, Tanno, et al. 1992). Cholinergic neurons are reportedly more susceptible to damage from trauma than other neurotransmitter systems (Schmidt and Grady 1995), and memory damage is associated with selective damage to hippocampal neurons (Hicks, Smith, Lowenstein, Sainte-Marie, & McIntosh, 1993).

Damage from the primary physical trauma is further compounded by secondary pathologies. The most serious secondary event is the brain edema that develops largely over the first 24 hours following the trauma (Klatzo 1967, Klatzo, et al. 1958, Saatman, et al. 1998). Once established there is no significantly effective treatment to permanently reduce the edema (Kimelberg 1995a, Kimelberg 1995b, Schilling and Wahl 1997, Shapira, et al. 1993, Wahl, et al. 1993, Wahl, et al. 1988). It is often associated with a period of coma or disorientation. The patient's condition improves slowly as the swelling begins to resolve.

The tissue swelling within the limited space of the skull creates increased intracranial pressure (ICP), which, in itself, can be life threatening. The elevated intracranial pressure (ICP) causes a reduction in cerebral blood flow (CBF) associated with a marked reduction in glucose and oxygen (ischemia) available to the traumatized and adjacent areas - causing additional cell death. The severity of disability following a head injury has been often more related to the degree of swelling than to the primary injury (Cervos-Navarro and Lafuente 1991).

Increased permeability of the blood-brain barrier (BBB) is a major factor in the formation of brain edema. Unique to the vascular system of the brain, the BBB stabilizes the extracellular environment within the brain, essential for normal neurological function, by limiting the free movement of water, ions, and most non-lipid soluble molecules in either direction. The increased permeability following trauma results in an influx of serum proteins and ions that disrupts osmotic homeostasis and facilitates movement of water into the brain tissue (Goldstein and Betz 1986, Kandel, et al. 2000, Kettenmann and Ransom 1995, Risau and Wolberg 1990). Observations by a number of investigators that edema development does occur over at least a period of about 24 hours implies a therapeutic time window (Baskaya, et al. 2000, Baskaya, et al. 1997, Dhillon, et al. 1994, Dux and Joo 1982, Hall 1993, Holmin and Mathieson 1995, Joo, et al. 1994, Preston, et al. 1993, Roof, et al. 1996, Shapira, et al. 1993). Extensive research on post trauma edema has provided considerable knowledge the pathophysiological mechanisms

involved. A treatment effective within a therapeutic time window to considerably reduce the edema would increase the potential for recovery and rehabilitation.

The majority of studies to work out the pathological mechanisms of head injury and brain edema have employed more severe experimental models. In recent years, a few studies have reported models of "mild head injury" (Dhillon, et al. 1999, Hicks, et al. 1999, Hicks, et al. 1993). This study used a new model of mild-to-moderate head trauma developed in this lab. In addition, it is significant to consider that brain edema is a serious secondary and damaging effect for occlusive strokes.

Mast cells contain numerous inflammatory mediators. However, mast cell-induced vascular permeability is considered largely due to histamine (Dimitriadou, et al. 1991). This decision was based on the large amounts available from dural mast cells compared to the normally low levels in the brain cortex (Goldschmidt, et al. 1985) and its potency as an inflammatory mediator.

Numerous studies in the literature have reported that histamine, in various dosages and applied via different routes, is associated with increased permeability of the BBB. Mast cells have been shown present in some areas on the brain, in particular, areas of the thalamus and along blood vessels. The largest and most dense concentration of mast cells is found in the dura mater, the meningeal layer directly adherent to the inside of the skull (Selye 1965, Selye, et al. 1963). Nevertheless, there has been a remarkable lack of association of the effects of histamine on the BBB, dural mast cells, and trauma-induced

degranulation. Consequently, a line of research with the potential of leading to a major interventional drug therapy to reduce brain edema has been neglected.

Most studies have found the histamine H_1 receptor antagonists alone to be of little to no therapeutic benefit. Some studies have found that H_1 antagonists enhance the ability of histamine H_2 receptor antagonists to reduce increased BBB permeability. This study elected to not use H_1 antagonists. These drugs have sedative side effects (Simons, et al. 1999, Yanai, et al. 1998, Yanai, et al. 1999). This would contradict its prescription by a physician for use in a head-injured patient because it would interfere with neurological status evaluation in the post-injury period. A number of studies using several models of altered BBB permeability leading to edema successfully reduced the edema using histamine H_2 receptor antagonists such as cimetidine and ranitidine. These H_2 antagonists do not penetrate the intact BBB and to block the H_2 receptors in the BBB vasculature the BBB has to first be compromised (Gross 1981). Many studies using experimental models of stroke have also shown several H_2 antagonists to have beneficial effects of limiting BBB permeability changes and subsequent edema formation. This further underscores the importance of pursuing this line of research.

A newer drug currently available for research only, is zolantidine. Zolantidine is a highly selective, potent H_2 antagonist that is also permeable to the BBB (Calcutt, et al. 1988, Gogas and Hough 1988, Simons, et al. 1999, Yanai, et al. 1998, Yanai, et al. 1999). Its primary application so far has been to study neuronal histamine H_2 receptor mechanisms and pathways in the brain and spinal cord (Calcutt, et al. 1988, Gogas and

Hough 1988, Gogas and Hough 1989, Gogas, et al. 1989, Hough and Nalwalk 1992, Hough, et al. 1990). This study elected to use zolantidine in an attempt to block as many H2 receptors as possible before the trauma in order to assess their role in these pathological mechanisms.

Evaluation of BBB integrity was by intravenous injection of Evan's blue dye into the mice. Evan's blue is non-toxic and binds to serum albumin, the smallest and most abundant protein in the blood. Albumin normally does not cross the blood-brain barrier. Its leakage into the brain tissue, along with the Evan's blue, has been widely used to assess increased permeability of the BBB (Clasen, et al. 1970, Del Zoppo 1988, Deli, et al. 1995, Dietzel, et al. 1969, Green 1990, Rawson 1943, Saria and Lundberg 1983, Saria, et al. 1983). For this study, a micro-spectrofluorometric assay was adapted to measure Evan's blue in the cortical tissue (Gross 1981, Rossner and Tempel 1966, Shapira, et al. 1993, Uyama, et al. 1988).

The hypothesis of this study is that mild to moderate head injury activates dural mast cells to degranulate, releasing large histamine. Some of this histamine diffuses across to the brain surface and mediates increased permeability of the BBB: essential to edema formation in subjacent cerebral cortex. This effect is mediated by histamine H_2 receptors and appropriate intervention with an H_2 antagonist will block this histamine effect. The aims of this research are to use a new model of mild to moderate head injury to document the association between dural mast cell degranulation and increased permeability of the BBB. Specifically, the first aim is to demonstrate a decrease in histamine content of the

dura subjacent to the trauma was associated with an increase in subjacent cortical histamine. The second aim is to determine if changes in histamine were associated with significant increased permeability of the BBB. Overall, the study wishes to evaluate whether or not blocking histamine H_2 receptors would reduce changes in the BBB permeability despite the trauma-induced degranulation of dural mast cells. Thus, the third specific aim is to evaluate whether blocking histamine H_2 receptors would reduce the severity of the changes in the BBB permeability.

This study has shown that trauma-induced degranulation of dural mast cells is a source of large amounts of histamine. Some of this histamine reaches the BBB at the surface of the brain and is associated with significant increases in BBB permeability. Zolantidine, a histamine H_2 receptor antagonist, was effective at the highest tested doses of 10 and 20 mg/kg in effecting a 65% reduction in the trauma-induced increased BBB permeability. Head trauma-associated brain edema is responsible for serious permanent neurological deficits for trauma survivors, whether severe TBI or multiple MTBI and there is currently no significant beneficial treatment available. The results from this research suggest that, given additional research to confirm and extend these findings, this study could lead to breakthrough treatments for reducing trauma-induced brain edema.

CHAPTER 2

LITERATURE REVIEW

Traumatic brain injury. Traumatic brain injury research has focused primarily on the more severe forms of head injury. This is understandable because of the seriousness of the immediate threat to the person's life and the relatively few treatment options available to physicians. Added to this has been the almost certain negative prognosis for returning to normal life as it was before the injury. Cumulative damage resulting from many milder head injuries has been most well characterized for many years in professional boxers and popularly known as the "punch-drunk" syndrome (Jordan 1987, Mendex 1995). Nevertheless, boxers were obviously more uniquely at risk for such damage than other people. An occasional MTBI was not considered serious. Any immediate symptoms went away in reasonably short time and the person appeared to be normal. The concern over the long-term cumulative permanent damage from repeated MTBIs has continued to grow (Kelly 1999). In particular, observations of sports participants have contributed substantially in this area. One study comparing amateur soccer players with amateur players of other sports found concussion specifically is associated with impaired performance in memory and planning functions (Matser, et al. 1999). This study followed another that studied professional soccer players that found

major neurocognitive deficits in the soccer players not present in professional players of a non-contact type of sport, such as swimming (Matser, et al. 1998). As the data continues to build on this subject (Baroff 1998, Collins, et al. 1999, Kelly 1999, Lovell and Collins 1998, Matser, et al. 1998, Powell and Barber-Foss 1999). The attitude of the medical profession and the public is becoming more serious. At the same time, it is now recognized that the same neurochemical damage mechanisms are activated with MTBI as with TBI, only to a lesser extent. We still have no treatment to offer to intervene in these pathological processes, nothing to ameliorate the inevitable subsequent damage. Only in recent years have a few studies been published using models of MTBI (Dhillon, et al. 1999, Holmin and Mathieson 1995, Nida, et al. 1995). Milder trauma models may make it possible to understand better the specific early pathological mechanisms to better test the effectiveness of specific treatments. This is more difficult with the more severe models.

Studies of trauma have used puncture (stab) wounds to the brain (Mohanty, et al. 1989), heat lesions (Edvinsson and West 1972), and cold (liquid nitrogen) lesions (Klatzo, et al. 1967, Mitchell, et al. 1979, Oishi, et al. 1983, Orr 1984, Raymond, et al. 1984). A heat stress model in rodents has been developed to study the associated cortical edema. This problem is serious for small children in a region of India during extremely hot weather (Sharma and Dey 1986, Sharma, et al. 1992, Sharma, et al. 1990). A considerable number of studies on the brain edema formation associated with strokes also exists. In this case the trauma is an internal one. Several experimental animal models have been developed

over the last 50 years or so, but no single model has been successful in replicating the clinical spectrum of closed traumatic head injuries seen in humans. The two models most favored in recent years have been the fluid percussion and the controlled cortical impact models (Nida, et al. 1995). Both of these models commonly include a pre-trauma craniotomy to expose the surface of the brain, with dura, to be impacted. Although this is to avoid the breaking of bone and bone fragments in the brain tissue the craniotomy itself is a trauma (Baskaya, et al. 1997, Holmin and Mathieson 1995, Jordan 1987, Mendex 1995, Roof, et al. 1997, Shapira, et al. 1993). Olesen first observed that craniotomy alone induced an increased permeability of the pial blood vessels to Na+-fluorescein and FITC-albumin. He also observed that application of histamine increased this effect (Olesen 1987). In a subsequent study using Evan's blue to assess the vascular integrity of the brain surface vessels (Orr and Stokley 1995) the effect of the craniotomy was verified. In addition, this study demonstrated that the change was associated with decreased histamine content in the dural area involved in the craniotomy and an increase in histamine content in the subjacent cortical tissue. This demonstrated that what had not even been considered a traumatic procedure (the craniotomy) was actually causing the same changes in the blood-brain barrier known to lead to cortical edema formation. As a result of the last experiments, the idea of scoring the surface of the skull as a model of mild-moderate head trauma was developed and applied in this present study.

Trauma injury mechanisms. Shear forces are thought to be cause of diffuse axonal injury where axons are disrupted at right angles to their longitudinal axes, but even these

effects may be delayed and thus likely to be secondary to the mechanical forces. TBI triggers cascades in interactive biochemical and metabolic changes that result secondary auto-destructive processes. These secondary injury responses are responsible delayed injury damage, which can be extensive. Mechanical damage to the cell membranes initiates release of polyunsaturated fatty acids with damage potential, especially arachidonic acid and its lipoxygenase and cyclooxygenase metabolites. Phospholipase products may act to chelate magnesium, which can depress the energetic state of the cells. Platelet-activating factor is produced. Phospholipid hydrolysis releases free radicals (Hall 1993) which, in part, can cause peroxidation of membrane phospholipids. Lipid and bio-energetic changes alone can significantly alter ionic homeostasis with influx of Ca^{2+} and Na^{+}. There are rapid increases in extracellular levels of excitatory amino acid transmitters, particularly glutamate. Activation of their receptors, especially the NMDA receptor, leads to large increases in neuronal intracellular Ca^{2+}. Elevations in a number of other transmitters have also been documented, such as acetylcholine and serotonin. Immune and inflammatory responses are extensive. This includes inflammatory mediators such as the prostaglandins and leukotrienes, platelet-activating factor, numerous cytokines (which in the brain also act as neurokins), and kinins. Microglia in the trauma area become reactive and a reactive gliosis occurs among the glial cells. Because the glial cells have such critical roles in neuronal homeostasis, including maintaining a favorable extracellular microenvironment for neuronal functioning, loss of

glial functioning greatly compounds the damage. There is a reduction in blood flow to the damaged area associated with ischemia.

Activation of mechanosensitive channels leads to an influx of Ca^{2+}, $Na^{+,}$ and Cl^{-} followed by swelling. Processes dependent upon adenyl cyclase may be directly and rapidly activated by forces encountered in TBI (Watson 1990). Hypoxia and ischemia will further exacerbate such swelling (Kimelberg 1995a). Mechanical distortion can also activate stretch (stress or distortion)-activated ion channels (Sackin 1994) including those in astrocytes (Bowman, et al. 1992).

Potential therapies aimed specifically at these mechanisms are being considered or tried. These include megadoses of steroids to inhibit lipid peroxidation (not very successful), antioxidants and free radical scavengers (vitamins E, C, A, and coenzyme Q, superoxide dismutase), antagonists of platelet-activating factor, inhibitors of the arachidonic acid metabolism (cyclooxygenase inhibitors), gangliosides (GM_1) (not very beneficial), glutamate receptor antagonists, especially to $NMDAR_1$, ion channel blockers (nimodipine, to block the Ca^{2+} -L channel) (O'Connor and Kimelberg 1993), and blocking the receptors for some of the most potent of the inflammatory cytokines, such a IL-1.

It is of note that the damage mechanisms discussed above are from consideration of the damage on the brain side of the BBB. Some of the above treatment approaches may be beneficial because many of these processes on ongoing after the trauma. The critical factor in development of extensive secondary damage is increased permeability of

the BBB and the resultant brain edema. Excitotoxic damage from excessive glutamate at NMDA receptors occurs extremely rapidly - within minutes. The associated influx of Ca^{2+} is so extreme that most of the affected cortical neurons will die (Giffard, et al. 1990). Without the physiological support from the glial cells the glutamate toxicity in magnified. The excitotoxicity generates a very high extracellular potassium (Pappas and Ransom 1994) level which acts to further depolarize additional cells. This paper ignores events at the BBB as well as any role of dural mast cells. The paper states that "inflammatory and immune responses begin within hours" (Faden 1996). However, in this study we have observed that degranulation of dural mast cells occurs almost immediately with the trauma. Elevations in cortical levels of histamine, which is also not mentioned, observed as early as 5 min in our mild-moderate trauma model. Likewise, the alterations in the surface vessels comprising the BBB can often be seen as early as 5 min following the trauma.

There are some promising reports of new approaches. Progesterone has been reported to greatly reduce the severity of edema formation and improve recovery of function in rats (Roof, et al. 1994, Roof, et al. 1996, Roof, et al. 1997). Two new drugs are being tested: riluzole (Wahl and Stutzmann 1999) and citicoline (Baskaya, et al. 2000), which show positive effects on modulating the BBB changes and edema formation and have a neuroprotective effect.

Edema. Brain edema refers to the pathological condition of brain swelling. Within the closed confines of the skull and meninges swelling of the brain leads to a life threatening

cessation of the brain's blood supply due to raised intracranial pressure (ICP). It causes displacement of and damage to brain tissue.

In the first two decades of the century, early researchers had already identified two forms of brain swelling following injury, stroke, or seizure. Brain edema was considered to be traumatic in origin and brain swelling was usually considered either toxic or metabolic. Throughout this time, the specific origins of edema were unknown. Klatzo sought to clarify the origins of edema. He eliminated the word swelling in favor of edema, defining it as "an abnormal accumulation of fluid associated with volumetric enlargement of the brain." One form was "vasogenic", in which there was injury to the blood vessel walls leading to escape of plasma constituents and water. The other he called "cytotoxic" edema, in which "a noxious factor directly affects the stranded elements of the parenchyma producing intracellular swelling; vascular permeability remaining relatively undisturbed".

General characteristics of these two forms of edema as defined by Klatzo are Vasogenic edema increased permeability of the blood-brain barrier (BBB), leading to net gain of fluid. Coincidentally cell swelling, mainly of astrocytes, is seen in gray and white matter. Cellular edema manifests as intracellular swelling without increased permeability of the BBB. It is seen mainly as a swelling of the astrocytes, but can involve swelling of myelin lamellae and dendritic swelling of neurons. Studies done since 1967 have indicated that the term cytotoxic edema is too restrictive, and cellular edema is now the preferred term.

Astrocytic swelling occurs in response to increased extracellular [K^+] (Kimelberg and O'Connor 1988, Pappas and Ransom 1994).

Vasogenic edema always involves a net gain of water and solutes. This is measured as a rise in intracranial pressure (ICP). The additional water and solutes enter the brain because of a compromised BBB. The BBB consists primarily of intercellular, occluding tight junctions between the endothelial cells of blood vessels and capillaries supplying the brain. The tight junctions exclude even small ions from the CNS, so that the only diffusion across it and into the brain is by lipid soluble substances. Transport systems are present for moving polar substances into and out of the brain (e.g. glucose and amino acids) (Kimelberg 1995b).

Cellular edema may accompany vasogenic edema in encephalitis, trauma, and stroke (Laterra and Goldstein 2000). Traumatic brain edema is considered to be a mixed form of brain edema, a combination of cellular and vasogenic edema (Baskaya, et al. 1997).

The BBB is responsible for building up and maintaining the special composition of the CNS extracellular fluid that is the prerequisite for adequate neuronal function. Morphological features of the Blood Brain Barrier are: 1. Complex interendothelial junctions, 2. Paucity of vesicular transport. (Dux, et al., 1988) found approximately four transport vesicles per μm^2. Other studies have indicated that these may be mostly pits instead of transport vesicles. 3. High number of mitochondria: In BBB forming cells the relative mitochondrial volume is around 10% of the whole cytoplasmic volume, which is several-fold higher than in non BBB vessels. 4. Symmetrical or asymmetrical distribution

of specific transport systems and ion channels. 5. Marked enzyme activity: A variety of enzymes demonstrated in BBB endothelial cells. The activity of the Na^+, K^+ - ATPase is approximately 500 times higher than in human umbilical cord endothelial cells. The functional characteristics of the BBB resemble those of a tight epithelium including: 6. High transendothelial potential and resistance: The transendothelial resistance is about 1500 to 2000 Ω /cm^2, usually higher in arteries than in veins.

Time course for development of edema. Shapira et al observed that extravasation of Evan's blue into the brain tissue was maximal at 4 hours after the injury, but a residual defect in the blood-brain permeability was detectable up to 4 days after the injury. A second opening of the BBB was not observed up to 7 days. The cerebral edema peaked at 24 hours in the injured and untreated rat. The edema resolved between four and 7 days. In the noncontused hemisphere Evan's blue increased at 15 min and 1, 2, and 4 h after the closed head trauma, but no tissue edema was detectable at any time in the non-injured hemisphere tissue (Shapira, et al. 1993). Dhillon et al., found that Evan's blue extravasation peaked at 3 h but was still high at 6 h post trauma (Dhillon, et al. 1999). Baskaya et al. observed a biphasic opening of the BBB after TBI in rats. Peak of cortical edema formation seen at 4-6 hours post injury and the barrier remained open 24-48 h in injured animals. At 3 days a second increase in permeability occurred. Brain edema first seen at 2 h following injury and in all brain regions at six hrs (Baskaya, et al. 1997). Roof et al. Observed a protective effect for progesterone against edema development with treatment delays of up to 24 h (Roof, et al. 1996).

Treatment of brain edema. Even a small net gain of fluid may lead to increase of ICP, which appears to be a critical step in the pathogenesis of secondary brain damage following trauma, stroke, intraparenchymal or subarachnoid hemorrhage, and tumor. Increase in ICP may result in a decrease of cerebral perfusion pressure, an impairment of the microcirculation and finally ischemia. Consequences of ischemia include breakdown of ionic homeostasis, cellular acidosis, and swelling and release of excitotoxic neurotransmitters and autocoids. Some of these autocoids may act on the BBB to further increase permeability and further increase ICP. A vicious circle develops until the structural integrity of neurons and glial cells is lost. To prevent brain edema is an important consideration when treating patients with intracranial pathology. Once the edema develops, therapy is primarily directed at controlling the ICP, maintaining it below a critical pressure of 20 to 25 mm Hg. The most commonly used measures to decrease ICP include head elevation and mild hyperventilation, drainage of CSF, application of hyperosmotic fluids mannitol or hypertonic saline (7.5%) solution (Horn, et al. 1999), induction of barbiturate coma and systemic hypothermia. Glucocorticoids: a decrease in BBB permeability seen after treatments with glucocorticoids in animals and cultured cells. However, in-patients a beneficial effect was observed only for people with tumor associated edema and then only with a decrease leakage from the tumor vessels. No beneficial effect was observed in trauma or stroke patients. Barbiturate coma: reduction of vascular tone, free radical scavenging action, decrease of cerebral metabolism. Systemic hypothermia: decreases cerebral metabolism Other therapies targeted to likely

effectors or secondary consequences: glutamate receptor antagonists, Ca^{+2} transport blockers, free radical scavengers of antioxidants, blocking anion channels or other anion transporters

Hyperbaric (1.5X) O_2 (HBO) therapy was used to attempt to reduce edema as measured at 6 h post trauma (Nida, et al. 1995). The HBO was not given until 4 h post injury and lasted for 60 min. Animals in the hypoxia group received 30 min of 13% O_2 immediately after the trauma. This study compared the results between animals receiving fluid percussion (FP) vs. cortical impact (CI) trauma. The craniotomy was performed 48 h before the trauma, which indicates the authors may have been aware of the effect of craniotomy alone although this goes unstated. It turned out that the hyperbaric O_2 therapy was effective in reducing edema in the fluid percussion model but not in the cortical impact model. The implications of this might be that the different models induce different neurochemical responses to trauma. The question might be if this difference is significant enough to warrant re-evaluation of the results of previous studies and if so, how is such re-interpretation approached.

Therapies directed specifically at reducing or blocking the increase in permeability of the BBB are not available yet. Therefore, treatment of brain edema is aimed at control of the ICP and protection of the neurons and glial cells at risk from secondary ischemia (Schilling and Wahl 1997).

Research has provided extensive knowledge of the complex pathological mechanisms and cascades of cellular events pertinent to them. Numerous treatments directed toward

these, often demonstrating significant beneficial effects in experimental laboratory animal studies has failed to similarly be equally effective in human clinical trials and application.

The most promising approach would be to develop therapeutic intervention to block or greatly reduce the edema development in the first place. Perhaps the major factor in brain edema formation is the increased permeability of the BBB. Treatments that could block the most potent major mechanisms responsible for increased permeability of the BBB would confer the greatest degree of protection.

A new drug, riluzole, which is designed to block Na^+ channels, has been shown to limit the size of the injury lesion and the edema is being tested. Riluzole can be beneficial within a time window of 1-6 h post injury (Wahl and Stutzmann 1999). Combination treatment of riluzole and mannitol has also been effective (Pratt, et al. 1999).

Histamine studies. Until the 1970's, histamine research almost completely focused on the role of histamine in allergic conditions and diseases. In 1966, Ash and Schild observed that some of the available antihistamines did not counteract all of the actions observed of histamine (e.g., in the heart and intestinal system) (Ash and Schild 1966). They proposed the existence of two subtypes of histamine receptors. Black et al., succeeded at synthesizing a group of antihistamines effecting at the receptors in the heart and intestines (brimmed, cimetidine) which soon became important tools in the treatment of gastric ulcers. Only more recently has the role of histamine as a neurotransmitter been documented (Schwartz, et al. 1991). As with other neurotransmitters, histamine has also been found to have a third receptor, H_3, which functions to control the synthesis/or

release of histamine at the axon terminal (autoreceptor) (Schwartz, et al. 1990). In other transmitter-specialized neurons, to control release of other neurotransmitters such as serotonin, dopamine, norepinephrine, or acetylcholine (heteroreceptor) (Schlicker, et al. 1994).

Both H_1 and H_2 receptors are G-protein coupled receptors (GPCRs).

The H_1 receptor has been associated with formation of IP_3 and DAG. HA induces IP_3 production via a pertussis toxin-insensitive G-protein. The G-protein probably belongs to the $G_{\alpha q}/G_{\alpha 11}$; its actual nature remains unclear. Studies indicate that H_1 activation of PLA_2 and cAMP elevation is mediated by an unknown secondary mechanism. The H_2 receptor appears most commonly coupled to adenylate cyclase. Evidence does exist for other coupling mechanisms. The H_3 receptor is thought to also belong to the GPCR family of receptors. Its mechanisms of second messenger coupling and signaling are not clearly defined yet. Information on the receptors comes from studies with many types of cells from different tissues and species, which adds to the difficulty of understanding the mechanisms by which these receptors function in the CNS.

The effects of histamine on vascular permeability vary with species-specific variation (Chang, et al. 1979) in sensitivity, tissue, characteristics of the local vasculature, microenvironment, receptors subtypes present and their distribution, and second messenger coupling. Other factors that can influence the response to histamine: other physiological factors and states such as those relating to circadian, immune, endocrine, and psychological events. Important also are the administration of other drugs during the

experiment, dosages, routes and schedule of administration. Not all drugs of a given subtype have equal potency and selectivity for the same receptor. Non-specific effects most frequently seen at higher dosages, may confound results, or produce opposite effects.

A transmural distribution for histamine receptors was suggested by the following results they observed: (a) intra-arterial injection of histamine caused increased BBB (endothelial cell) permeability via H_2 receptors, (b) increased blood flow in response to intra-arterial histamine occurred only after the BBB was compromised and resulted from activation of both H_1 and H_2 receptors. These responses probably occur within inner layers of arterial smooth muscle.

Dilation of pial arterioles to local micro-application of histamine and its receptor agonists indicates H_2 receptors are the predominant type in the outer layers of arterial smooth muscle.

Within the cerebrovascular bed, a segmental profile of histamine receptors is suggested as follows: (a) since both H_1 and H_2 receptors could mediate dilation of arterioles and arteries, both types of receptor are present in resistance vessels, (b) in the capillary bed H_2 receptors are the predominant type. Capacitance vessels (pial veins) did not respond to perivascular application of histamine or its agonists.

H_1 receptors are located predominantly along the inner layers of vascular smooth muscle and mediate vasodilatory responses to intra-arterial histamine (Powell and Brody 1976). H_2 receptors, with greater concentrations in the outer vascular smooth muscle,

mediate the responses to mast cell histamine (Powell and Brody 1976) and to histamine applied to the outside of arteries (Galeno, et al. 1979).

Infusion of histamine into internal carotid artery for 15 min increased permeability of labeled sucrose 2X, an effect completely blocked by prior administration of a H_2 antagonist (metiamide) but not by a H_1 antagonist (mepyramine) (Gross, et al. 1981a). Gross also made the correct association between the increase in adenylate cyclase activity and the actions of H_2 activation (Gross 1981).

Histamine infusion intra-arterially causes vasodilation by different receptors in different tissues. Gross et al (Gross, et al. 1981a) determined that, in rats, carotid artery infusion of histamine by itself had no effect on cerebral blood flow, caused no vasodilatory responses. If, however, the BBB was first disrupted by hypertonic solution infusion, HA caused modest increases in cerebral blood flow that could be blocked by either H_1 (mepyramine) or H_2 (metiamide) antagonists. The increased blood flow appeared to result only from vasodilation because increases in cerebral glucose consumption were not detected (Gross, et al. 1981b).

Application of H_1 and H_2 agonists perivascularly to the pial vessels produced vasodilation to the H_2 agonists but only a weak response to very high concentrations of the H_1 agonist. This indicated that although the H_1 receptors were present they did not play a very important role in the vasodilatory response to histamine. The response was predominantly mediated by H_2 receptors present in outer layers of cerebrovascular

smooth muscle (Gross, et al. 1981c). It was found that the pial veins did not respond to histamine, H_1 agonists, or H_2 agonists.

Histamine decreased electrical resistance in both arterial and venous microvessels similarly. Addition of 10^{-4} histamine resulted in a 75% reduction in the electrical resistance, in both arterial and venous vessels, indicating a marked permeability of the blood-brain barrier. The histamine dihydrochloride was added to the ACSF perfusing the brain surface. Cimetidine completely blocked the histamine-mediated increase in BBB permeability, whereas promethazine had only a small effect, and indomethacin was completely ineffective. In addition, cimetidine treatment resulted in a 100% increase in basal resistance in both arterial and venous blood vessels. This suggested endogenous histamine was acting to increase blood-brain barrier permeability. The conclusions were that histamine causes an increase in BBB permeability mediated by endothelial H_2 receptors. Pretreatment was given 20 minutes before the surgical procedure by i.p. injection (Butt and Jones 1992).

The effect of antihistaminics on experimental brain edema in rats. Circular craniotomy exposed brain surface with dura intact. A cold (-75°C) copper cylinder contact for 60 s. Measurements of water content, and Na^+ and K^+ to evaluate edema. Tested zolantidine and mepyramine and combination of the two. Drug injections were i.p. every 6 h, beginning 24 h before the procedure. Observed no reduction in brain edema associated with treatment with the antihistamines (Schilling and Wahl 1994b).

Cortical superfusion with histamine induced a concentration-dependent leakage of the tracers. Leakage started from venules and small veins. Used tracers: Na^+-fluorescein (MW 376), and fluorescein isothiocyanate (FITC) labeled dextran (MW 62,000 or 145,000) Intravital fluorescence microscopy. For Na^+ fluorescein leakage began with histamine at 10^{-9} M and was 100% at 10^{-7} M. For FITC-dextrans 62,000 and 145,000 leakage started at 10^{-8} and 10^{-6} M, respectively. They observed that impromidine was 54X more potent than histamine. They also observed, in these experiments, that cimetidine did not reduce the leakage of these tracers in response to histamine application. They observed that cimetidine itself caused a 12% extravasation of the tracers in the absence of histamine. After 60 min and superfusion with cimetidine at 10^{-6} and 10^{-4} M the extravasation was 50% and 65%, respectively, and remained constant during the following 60 min. Mepyramine induced similar results. Histamine opens the BBB for small and large tracers non-specifically (Schilling and Wahl 1994a).

Dropp (Dropp 1972, Dropp 1976) quantified brain mast cells and suggested that each might contain 20 pg of HA, the amount in peritoneal mast cells. This appears to be an over estimate. Mast cells in different areas contain different amounts of histamine. This study determined that MCs in the (rat) thalamus correlated well with the number of thalamic MCs. Results indicated that MC numbers accounted for 71% and 46% of the variation in thalamic HA in males and females, respectively. The thalamic MC numbers also correlated significantly with whole brain HA levels. Thalamic MC numbers account for 35-39% of the variation in whole brain HA levels. Thalamic MCs contribute 30% and

20% of whole brain HA levels in males and females, respectively (a range of 0%-50% for both sexes). Other species and strains likely will show other differences. Taking into account the amount of HA measured and the numbers of MCs, brain MCs probably do not have near the 20 pg found in peritoneal MCs. These authors estimate rat brain MCs have between 1.3 and 2.5 pg HA/cell (Goldschmidt, et al. 1985).

Mast cells. In 1863, Friedrich von Recklinghausen recognized granular cells in the unstained mesentery of the frog, which Paul Erlich, in 1878, after staining, called "mast cells". The mast cell represents a fixed storehouse of potent biologically active substances. Mast cells have been called pharmacological "time bombs" (Pepys and Edwards 1979). Hans Selye wrote an extensive review encompassing 2,500 publications on mast cells in 1965 (Selye 1965).

Mast cell degranulation and staining: Triggers for MC degranulation include mechanical and thermal stimuli, allergic mechanisms, $Fc_{\varepsilon}RI$ receptors, IgE-mediated immune mechanisms via $Fc_{\varepsilon}RI$ receptors, and chemical activators such as: compound 48/80, polyamines, polymixin B, and carbachol. Myelin Basic Protein (MBP) triggers activation of MCs and MC proteases participate in demylination in central and peripheral nervous systems.

Mast cell mediators are numerous. Although the profile of mediator compounds a given mast cell may contain can vary according to its tissue location, developmental stage, and state of activation. The range of contents for mast cells includes: histamine (HA), serotonin (5-HT), platelet activating factor (PAF), prostaglandin D2 (PGD_2),

leukotrienes C_4 (LTC_4) and D_4 (LTD_4), bradykinin (BK), proteases, glucuronidase, phospholipases, thromboxanes, heparin, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), nerve growth factor (NGF), transforming growth factor β, interleukins: IL-1, 2, 3, 4, 5, 6, chemotactic factors for neutrophils, basophils, eosinophils, and monocytes.

Arachidonic acid increases capillary permeability in dose dependent manner up to 2 mM; higher doses, up to 5 mM produced no additional increase in permeability and caused profound tissue destruction around the needle tract. The increase in permeability occurred within 2 h after perfusion and continued through 24 h. The effect of 48 h infusion was half that at 24 h Mechanisms suggested (1) direct detergent effect of arachidonic acid, (2) the effect of arachidonic acid that is released from the membrane by the activation of phospholipase A_2. Pre-treatment with dexamethasone significantly inhibited the arachidonic acid-induced increase in capillary permeability. Pretreatment (1 h) with actinomycin D suppressed the inhibitory effect of dexamethasone (Ohnishi, et al. 1990).

Serotonin, and BBB, 5-HT administered, i.v, at 10^{-6} to 10^{-4} M, reduced electrical resistance in frog pial venules. Effect blocked with 5-HT antagonist ketanserin (effect on 5-HT_{2A} receptors, and to a lesser degree on 5-HT_1 receptors.) (Note: DOI is agonist for 5-HT_{2A} receptors), or by verapamil. Conclusions: A 5-HT_2 mediated influx of Ca^{2+} is followed by endothelial cell “contraction” resulting in opening of the tight junctions.

(suggested). 5-HT by this mechanism can induce penetration of small ions but not larger species. Na^+-fluorescein, for example, did not leak through (Olesen 1987).

TNF-α, is produced, stored, and released by mast cells. TNF-α enhances capsaicin sensitivity of sensory neurons, and can contribute to development of hyperalgesia. TNF-α and IL-1β are released by injury and are critical in development of the inflammatory cascade. They also enhance release of arachidonic acid and eicanasoid synthesis, especially PGE_2 by induction of PLA_2 and cyclooxygenase activities. This elicited release of PGE_2 can sensitize cells and potentiate responses to other inflammatory agents, such as bradykinin. Sensitization is accompanied by increased firing rate of sensory axons.

Phospholipase A_2 (PLA_2) activity leads to increased release of arachidonate and its metabolites.

Prostaglandins (PGs) enhance the ability of other substances to cause plasma extravasation. PGs are produced and released by sympathetic neuron terminals. Inhibitors of PG synthesis inhibits PE, and also hyperalgesia caused by bradykinin .

PAF causes platelet activation. Platelet α-granules are storage site for most of the proteins released upon platelet activation, e.g., PDGF, TGF-β, RANTES, and IL-7.

Histamine-releasing cytokines, (cytokines with histamine releasing activity, independent of IgE mechanisms): C - C chemokines such as MCP - 3, MCP - 1, RANTES; also, IL-3.

IL-1, triggers MC degranulation. IL-3 functions as a mast cell growth factor. Astrocytes synthesize IL-3, a cytokine that can stimulate release of histamine from mast cells.

Nerve Growth Factor (NGF) modulates sympathetic innervation (Carlson, et al. 1995) and maintains sensitivity of mature sensory C-fibers (Leon, et al. 1994, Levi-Montalcini, et al. 1996) NGF also increases production of and surface expression of clathrin, and increases recycling of clathrin (Beattie, et al. 2000).

CRH is associated with activation of sensory C-fibers in the dura (70%) (Theoharides, et al. 1995).

The metachromatic staining with certain basic dyes such as toluidine blue is due to the presence of acid mucopolysaccharides in the granules. The removal of basic histamine most likely makes acidic groups available for dye interaction. Mast cell granules contain a matrix of heparin and proteins for the binding of mast cell amines (Lagunoff, et al. 1964).

MCs not stimulated to secrete have a surface characterized by anastamosing folds of cell membrane of equal depth and width. During exocytosis, these were replaced by deep cup-shaped flaps of membrane. With extensive exocytosis, plasma membrane folds were totally missing over large areas of the cell surface and circular or ovoid holes could be seen. Over the hours after exocytosis the flaps of membrane fused with other flaps or with the plasma membrane of the cell (Bytzer, et al. 1981). The degranulation process is Na^+-dependent (Lagunoff 1972a) and ATP dependent. The secretory process starts with a series of membrane fusions forming deep channels of extracellular medium into the MC. The granules are extracellular but retained within the cell domain (Lagunoff 1973). The process advances inward. Some granules undergo exocytosis resulting in release of both

amines and heparin into the extracellular environment. Amines are released on exposure of the granules to extra cellular cations (Bloom and Chakravarty 1970, Lagunoff 1972a). Granules in contact with the extracellular environment have altered morphology whether retained within the cell or not (Bloom and Haegermark 1965, Lagunoff 1973). Granules retained within the cell that release their histamine retain their heparin (Berlin and Enerbach 1984). The degranulation process does not result in complete disruption of the cell, nor are all the granules necessarily involved in a single secretory act. Ruthenium red has been used to stain extracellular but not intracellular mast cell granules. In non-secreting mast cells, few granules stain with this dye. In a MC with secreting granules extruded granules, whether completely extracellular or retained within extracellular channels within the domain of the cell, of the cell, bind the ruthenium red (Lagunoff 1972b). Amine contents of the granules, such as histamine, are released with heparin and with N-acetyl-β-glucosaminidase. At the same time, MC ATP, lactate dehydrogenase, and K^+ are not released with histamine. MCs maintained in isotonic sucrose solution and exposed to degranulating agents extrude granules but do not release histamine. If Na^+ is added to the extracellular solution, histamine is then released from the granules. The extension of the extracellular space by channel formation was confirmed using ferritin as an extracellular marker (Lagunoff 1972a, Rohlich, et al. 1971). There is an energy requirement involved in translocating the granules to the surface membrane. The second requirement is for a cation exchange (bicarbonate out for Na+ in) (Thon and Uvnas 1967, Uvnas 1963).

Frequently some MCs are observed with several vacuole-like formations, which often contain what appears to be a swollen MC granule and which stains bright red or pink. When they are seen they are always close to a cell and always stain pink. Sometimes MCs are seen with a granule, or an aggregate of granules that appear attached to the outside of the cell. Many cells that appear normal will have, on close examination, one or several vacuoles staining pink. Pink granules may appear scattered diffusely between cells In some instances mast cells may appear totally disrupted (Bloom and Haegermark 1965).

Mast cells associated with the CNS have been known for over 100 years. Neuman identified them in brain infarcts and multiple sclerosis plaques. (Neuman 1890). Mast cells in the brain are localized along blood vessels associated with the thalamus, hippocampal formation, corpus striatum and corpora quadrigemini and the choroid plexi of the lateral ventricles (Orr 1988b). MCs are also found along vessels associated with the dorsal root ganglia and spinal roots (Orr 1988b). Within the brain itself mast cells are present in significant numbers only in certain areas of the thalamus (Dropp 1976, Goldschmidt, et al. 1984, Ibrahim 1974, Olsson 1968). Mast cells have also been described in blood vessels of the leptomeninges and in large numbers in the dura mater (Dropp 1972, Dropp 1976, Edvinsson, et al. 1977, Irman-Florjanc 1997, Olsson 1968, Orr 1984, Orr 1988a, Selye, et al. 1963). The anatomical position of CNS mast cells in close apposition to major arterial vessels as well as perivascular and subarachnoid spaces

places them in the perfect position of facilitating communication between the CNS and neuroimmunoendocrine signals from the periphery or from elsewhere in the CNS.

Some MCs appeared to be freely distributed in dural tissue, many were positioned in association with the middle meningeal neurovascular bundle (Keller, et al. 1989). The Dimlich et al., 1991 study found there were two populations of dural mast cells: one associated with each of the two layers of dura they identify. The two groups of MCs had different shapes but were the same type of MCs. Mast cell shape is dependent on contiguity, density, and orientation of its surrounding elements. MCs in the outer layer were aligned parallel to the middle meningeal artery (MMA). Those in the inner layer were aligned parallel to trigeminal nerve branches obliquely crossing the MMA. In cross section it was observed that most MCs were aligned at the interface between the two dural layers (Dimlich, et al. 1991). The outer layer contains the neurovascular bundle that includes a central artery, two veins, and two nerve bundles. The inner layer contains a branch of the trigeminal nerve. There is a very thin layer of dense connective tissue at the outermost border and at the innermost border. Mast cells are distributed at the interface between the two layers as well as linearly on either side of the vessels and/or nerves in each layer.

Mast cells have been known to circulate as committed precursors rather than as mature calls (Galli 1993, Kitamura, et al. 1978). Under specific physiological conditions mature MC numbers in the brain increases rapidly (within 1-2 h), which can only be attributable to migration/translocation of mature mast cells from an extra CNS location into the brain.

A recent study has demonstrated this migration does occur and that the MCs transit the endothelium of the brain microcirculation (Silverman, et al. 2000). This has already been established for other immune system cells active in immune surveillance. Antigenic factors stimulate MCs migration in the periphery (Gruber, et al. 1994). MCs, express integrin pairs associated with homing of T-cells, LPAM-1, and-2, and can adhere to endothelial surfaces and exhibit a rolling behavior in a P-selectin-like manner (Yong, et al. 1995), considered the first step in diapedesis for other types of cells (Bebo, et al. 1996, Collins 1995, Green, et al. 1993).

Mast cell-neural interactions. Contacts between nerve terminals and mast cells closely associated with arterial vessels have shown three types of varicosities. (1) Some contain small dense core vesicles. Degenerates after superior cervical ganglionectomy, which leads to degeneration of the sympathetic innervation to cerebrovascular supply. The other two types remain unchanged after sympathectomy. (2) The majority of the remaining varicosities contain small clear vesicles which are characteristic of parasympathetic innervation. (3) The third group of varicosities contain only large dense core vesicles (usually described as peptide-containing vesicles) and probably belong to the peptidergic sensory nerve fibers originating in the trigeminal ganglion (Zhang, et al. 1993).

Sensory innervation of the dura is from the trigeminal ganglia. Trigeminal axon terminals innervating the cerebral blood vessels have large dense-core vesicles (100-120 nm diameter) which have been shown to store Sub P, and to co-store CGRP. These are

mostly unmyelinated C-fibers. Sensory neurons can release transmitters from their peripheral endings, and so have a local "efferent" function. The endings release neuropeptide that include Substance P (Sub P), neurokinin A, and calcitonin gene-related peptide. Other peptides, which may be co-stored and released, are somatostatin, vasoactive intestinal polypeptide (VIP), galanin, and corticotrophin-releasing factor (CRF). Polymodal nociceptors (C fibers) can be stimulated by a variety of agents, including inflammatory mediators such as histamine, bradykinin, and prostaglandins, as well as by capsaicin (Barnes, et al. 1990). Sub P and CGRP trigger mast cell degranulation (Ottosson and Edvinsson 1997) and can lead to an inflammatory process. Activation of the trigeminal fibers causes vasodilation of extra-cranial and intracranial, dural blood vessels and increases in vascular permeability. Electrical stimulation of the trigeminal ganglion induces MC degranulation in the dura mater (Dimitriadou, et al. 1991). This stimulation also has been shown to induce neurogenic inflammation in the dura mater attributed to the release of Substance P and CGRP by the trigeminal nerve endings (Buzzi, et al. 1992, Dimitriadou, et al. 1992, Dimitriadou, et al. 1994). CGRP is a potent vasodilator but does not directly increase vascular permeability. CGRP potentiates the effects of Sub P by inhibiting its breakdown. CGRP is the most potent vasodilator within pial blood vessels. Both Sub P and CGRP trigger degranulation of mast cells and this effect is synergistic. Sub P triggers MC histamine release via NK_1 receptors. C-fiber evoked-plasma extravasation (PE) is dependent on mast cells (Coderre, et al. 1989, Jansco, et al. 1967, Lembeck and Holzer 1979) but mast cell-evoked PE is not dependent

on C-fibers (Coderre, et al. 1989, Gamse, et al. 1980, Jansco, et al. 1967, Lembeck and Holzer 1979). CGRP has mast cell activating ability but is several-fold less potent in doing this than is Substance P. CGRP (10 μM) evoked a maximal release of 33% of the total MC histamine content. The CGRP effect was blocked with sumatriptan. The CGRP receptor antagonist $CGRP_{8-37}$ blocked the CGRP-evoked mast cell histamine release, and this suggests that CGRP is triggering the histamine release via $CGRP_1$ receptor-coupled activation of dural mast cells. The effects of the antagonists were concentration dependent.

Elevated levels of Sub P are associated with stress and anxiety. Intra-cerebroventricular (icv) infusion of Sub P in rats elicits an integrated cardiovascular, behavioral, and endocrine response pattern consistent with a stress response pattern for rodents. Sub P appears to serve to amplify ongoing immune reactions and as a mediator in stress-induced immune reactions (Brodin, et al. 1994, Fasmer, et al. 1983, Fehder, et al. 1997).

Mast cells produce nerve growth factor (NGF). NGF is important in maintaining normal levels of Sub P in mature sensory neurons. In the absence of NGF, the levels of Sub P stored and released by the sensory terminals is low, and the sensitivity of the nerve endings is reduced (Leon, et al. 1994).

There are several non-neural sources of Sub P. Macrophages synthesize and secrete Sub P (Pascual and Bost 1990). Macrophages from humans and guinea pigs have receptors for Sub P releasing cytokines: IL-1, IL-6, and TNF-α. Eosinophils (Weinstock,

et al. 1988) and vascular endothelial cells of several mammalian species, including humans, also produce Sub P (Linnik and Moskowitz 1989).

Sympathetic nervous system innervation contributes to plasma extravasation (PE): it is vasoconstrictive but increases vascular permeability. Norepinephrine (NE) typically inhibits PE or edema associated with inflammation. Neuromodulators colocalized with NE are neuropeptide Y (NPY) and ATP. Prostaglandins (PGs) are also released by sympathetic neuron terminals Inhibitors of PG synthesis inhibit PE and hyperalgesia caused by bradykinin. NPY causes post-synaptic inhibition at sympathetic neurons largely by inhibiting a N-type voltage-dependent calcium channel (VDCC) at the axon terminal. Effects of NPY at the sympathetic neuro-effector junction are two: (1) a presynaptic inhibition mediated by the Y_2 receptor, and (2) a potentiation of the post-synaptic actions of NE and other excitatory transmitters via the Y_1 receptor. Inhibition of NE release by Y_2 receptors - important in limiting the amplitude of the post-synaptic response or the desensitization of adrenoreceptors during elevated or prolonged activity. NGF produced by mast cells also modulates sympathetic innervation.

In smooth muscle cells, NPY causes a potentiation of L-type voltage-dependent calcium channels (VDCCs). This provides an increase in "synaptic gain" by amplifying post-ganglionic output. NPY induces mast cell degranulation, but not PGD_2 release.

CHAPTER 3

MATERIALS AND METHODS

Centered Subheading

Animals. SJL/J male mice (Harland Sprague Dawley, Indianapolis, IN) aged 2 to 3 months were used for the histamine determination experiments. SJL/ J female mice (Harland Sprague Dawley, Indianapolis, IN) aged 5 to 7 months were used for the Evan's blue permeability study and the mast cell counts. SJL/J female mice (Jackson Labs, Bar Harbor, ME) aged 2 months were used for the zolantidine experiments. The mice were housed in the Animal Care Facilities of the University of North Texas Health Science Center (UNTHSC), in standard clear plastic cages. Male mice were housed two to a cage and separated with a metal partition. The female mice were housed five to a cage. Food and water were provided *ad libitum*. The mice were kept under a light–dark cycle of 12 hours on (7 A.M.) and 12 hours off (7 P.M.). Temperature was maintained at 21-22° C and humidity at 50–60 %. After arrival, the mice were given a minimum of 7 days to acclimate before any experimental procedures were performed. Exposure to unnecessary stressful stimuli before the experiments was minimized. Surgeries were performed between 9 A.M. and 5 P.M. Experimental procedures were approved by the UNTHSC

Institutional Animal Care and Use Committee and were in accordance with the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research.

Chemicals. The following chemicals were obtained from suppliers as indicated. Methoxyflurane (Metofane, Mallinckrodt Veterinary, Inc., Mundelein, IL), isoflurane (IsoSol, Abbott Labs, North Chicago IL; distributed by VEDCO, Inc, St. Joseph, MO), surgical anesthetic gases (BOK Gases, Fort Worth, TX); Evan's blue, sodium phosphate monobasic monohydrate A.C.S., sodium phosphate dibasic anhydrous reagent A.C.S., trichloroacetic acid A.C.S., perchloric acid 70% A.C.S., formalin 37-40%, ScintiSafe™ Econo F scintillation cocktail, chromatography solvents: methanol, chloroform, sodium hydroxide solution, (Fischer Scientific, Houston, TX); Toluidine Blue, ninhydrin, histamine (2-[4-Imidazolyl] ethylamine) dihydrochloride, 1-Methyl-4-[β-aminoethyl] imidazole dihydrochloride (Sigma Chemical, St. Louis, MO), *S*-adenosyl-L-[methyl-^{3}H]methionine (Amersham Life Sciences, Arlington Heights, IL), and ethanol 200 proof, pharmaceutical grade (McCormick Distilling Co., Brookfield, CT).

Surgery. Anesthesia was with methoxyflurane or isoflurane. Methoxyflurane was administered by inhalation with 5% CO_2 and 95% O_2. Following induction of surgical anesthesia, a nose cone was used to maintain anesthesia through the remainder of the procedure. Isoflurane anesthesia was delivered using 100% O_2 with equipment usage and settings according to animal care staff instructions. Following induction of anesthesia, a nose cone connection to the anesthesia machine maintained a consistent level of anesthesia and oxygenation through the remainder of the procedure. The mice were

placed on a heating pad (Micro-Heat microwavable heating pad, Cara Inc., Warwick, RI) maintained at 37-38° C to prevent hypothermia (Brown 1997, Daniels 1991, Foster, et al. 1983, Kaibara, et al. 1999).

Evan's blue in physiological saline (0.89% NaCl) was prepared the day of the experiment and filtered with a 0.2 μm syringe filter (Corning Glass Works, Corning, NY). Following the method of Uyama, et al., 1988, 0.1 ml Evan's blue 2% w/v was injected intravenously (i.v.) into a tail vein 5-10 min before scoring of the lines. This procedure was used for all experiments except the zolantidine study. During the zolantidine experiments the Evan's blue dye was first injected, followed by subcutaneous (s.c.) administration of the zolantidine within 5 min. A 30 min period after the drug injection was allowed before the line scoring was performed.

The head trauma was produced by scoring of two lines in the dorsal parietal bone. Surgical scissors were used to make an incision in the skin of the lower neck, approximately 1-cm behind the ears, then extended left and right of the midline. A forward midline incision was made to just past the eyes. The skin flaps were pulled back and a small scalpel was used to scrape the surface of the skull to remove the periosteal connective tissue. A Dremel Moto-tool with a Moto-flex attachment (Dremel, Racine, WI) at a speed setting of 2-3 was used to cut the two lines. The diameter of the Dremel cutting blade was 23 mm but only 2 mm actually touched the surface requiring moving the blade back and forth to produce the proper lines. The area of the dorsal parietal skull was 30 mm from the sagittal suture midline to the outer downward curvature of the bone

and 40 mm between the interparietal and frontal sections of the skull. The lines cut into the skull but not through, the bone and dura (Fig. 1A). The exposed surgical area was covered with saline-moistened gauze and the mouse kept under anesthesia for the duration of the designated post-trauma survival period. The chest cavity was then opened and the right atrium snipped. Transcardial perfusion was performed manually via the left ventricle with 30 ml of warmed physiological saline. In experiments, involving determination of Evan's blue levels, a blood sample of 0.05 ml of blood was collected before the saline perfusion.

Scissors were used to decapitate the mouse. The skull cap and brain were removed by two lateral incisions made with surgical scissors inserted through the foramen magnum on each side and united between the eyes just back of the nose. The skull was separated gently from the brain and a visual examination made for hyperemia, hemorrhages, and Evan's blue extravasation. Sketches were made of the observations. A 4-mm diameter cork borer was used to take two plugs of cortical tissue from first the right hemisphere in an area correspondent to the left (experimental) hemispheric damaged area. As much as possible the myelinated white matter on the underside of the plug was removed. In experiments in which Evan's blue had been injected into the mouse, examination of the left surface of the cortex usually showed bluing of the surface corresponding to the position of the lines in the skull (Fig.1B). The tissue was placed in 1.5 ml microcentrifuge tubes, weighed and frozen at –20° C. Right and left duras were individually separated from the skull and saved in the same manner. Duras not used for

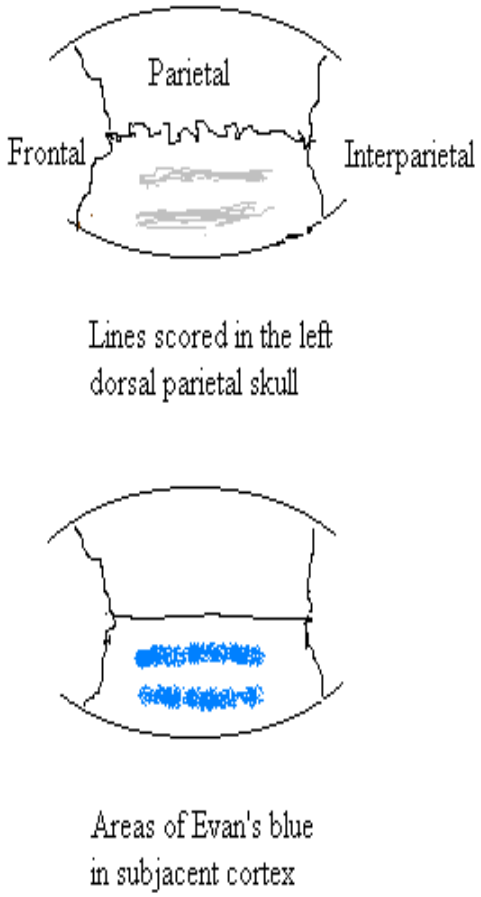

Fig. 1.
Illustration showing positioning of the (A) lines scored into the left parietal skull with the Dremel Tool. (B) The corresponding position of the blue coloration on the skull surface representing leakage of Evan's blue-albumin from the blood vessels into the brain tissue.

histamine analysis were left attached to the skull and were fixed in 10% neutral buffered formalin (NBF) for a minimum of 72 h. The flat mount procedure for the duras generally followed the "Periosteal Spread Technique" as described by Selye, et al., 1963.

Toluidine blue staining of dural mast cells. The skull with attached dura was rinsed in tap water to remove residual fixative. The dura was then stained with toluidine blue 0.5% in distilled water *in situ* in the skull for 3-min. Staining was followed with 3 rinses in tap water acidified with HCl to pH 3.0. Excess water was drained away and the dura separated from the skull with the blunted tip of a metal probe. The dura was floated in a dish of water and onto a glass slide (25 X 75 X 1 mm) (FisherBrand™, Fisher Scientific, Houston, TX) where a flat spread of the tissue could be made. The preparation was covered with either glycerol or Fluor-Save™ (Calbiochem-Novabiochem, La Jolla, CA) and a glass coverslip (22 X 30 X 0.5 mm) (FisherBrand™, Fisher Scientific, Houston, TX) for microscopic examination.

Mast Cell Counts This study counted the degranulated/activated mast cells and non-degranulated/unactivated mast cells in both the left and right duras. For these experiments only a single line was scored instead of the two. The reason for using a single line was to make it easier to determine differences in MCs degranulation the two time points. Toluidine Blue staining was followed by counting through a Zeiss phase contrast microscope and 25X objective. Photos were taken of examples of normal/unactivated and degranulated/activated mast cells using an Olympus 35-mm camera back, Model PM-6 (Olympus Optical Co., LTD, Tokyo, Japan). Photos were at 40X, 100X, and 160X using

Kodak Gold 100 film. Film prints were by Wal-Mart and Walgreen's One Hour processing. Additional black and white photography were made using a Nikon Microphot - FXA with a CDC digital camera. Photographs were stored on a computer and photo files managed with a UNTHSC proprietary software program.

Histamine Assay. Cortical tissue (20–40 mg) was homogenized in distilled water with a microliter volume of 4X the weight of the tissue (mg wet wt). Dural tissue was homogenized in 500 µl of distilled water. All tissue samples were homogenized for 30 sec each using a Kontes micro-ultrasonic cell disrupter (Kontes, Vineland, NJ). The samples were placed in a boiling water bath (100° C) for 10 min to destroy any endogenous histamine-*N*-methyltransferase (HMT) (stock preparation by the method of Shaff and Bevan (Shaff and Beaven 1979), and *S*-adenosyl-L-methionine (SAM). Samples were cooled on crushed ice following the boiling water bath then centrifuged at 16,000 g for 10 min at 4° C in a Sorvall ultracentrifuge (Sorvall, Inc., Newtown, CT).

The radioenzymatic histamine assay was performed as previously described (Orr and Pace, 1984). Duplicate 10 µl aliquots of the tissue supernatants were added to two 400 µl microcentrifuge tubes on ice. Tubes for blanks had cold distilled water added in place of supernatant. Two additional 10 µl aliquots of each supernatant were run with duplicate 10 µl aliquots of a histamine standard that were prepared in 0.5 *M* phosphate buffer (0.05 *M* Na_2HPO_4, pH 7.9). Histamine standard solutions of 50, 100, 200, and 400 pg from the assay were used to produce a standard curve based on internal standards. Assay tubes not receiving 10 ml of a standard in buffer received 10 µl of the buffer added. The distilled

water blanks were always run with buffer alone. A reaction mixture containing 2 µl HMT, 1 µl (1 µCi), ^{3}[H]-SAM, and 7 µl phosphate buffer was added to each tube. After a brief spin (several s) in a Beckman Microfuge B, the tubes were left at room temperature for 1 h. The tubes were then placed on ice and 10 µl of stop mix (1 mg *tele*-methylhistamine hydrochloride /1 ml 0.4 *M* perchloric acid) was added to each tube. Following another brief spin in the microfuge, the tubes were returned to the crushed ice for 20 min. During this time the thin layer chromatography (TLC) plates (LK50 Silica Gel, Whatman Laboratory Division, Clifton, NJ) were activated in a dry oven (100° C) for 20 min. The tubes were centrifuged in the microfuge for 10 min. The cooled TLC plates were then spotted with 20 µl of supernatant onto individual lanes of the TLC plate. The center lane of each plate was spotted with 5 µl of the methylhistamine stop mix. The plates were air-dried at least one h in a fume hood. During this time chromatography solvents (chloroform, 60 ml; methanol, 35 ml; and ammonium hydroxide, 5 ml) were added to the vat. New sealant grease (NuSil Technology, Carpinteria, CA) was applied along the top edges of the vat. The prepared vat was placed in a refrigerated cold box. The plates were placed evenly into the solvent mixture in the vat and the rat returned to the cold box. Development of the plates required 1.75-2 h. The plates were removed when the solvent reached the scribed line at 13 cm and dried in a fume hood for a minimum of 45 min to 1 h. The plates were sprayed with ninhydrin (0.2 g/100 ml EtOH). The ninhydrin was applied with a CHROMIST™ SPRAY UNIT (Geldman Instrument Company, Ann Arbor, MI). The plates were placed in the dry oven (100° C) for 5 min to

develop the coloration of the methylhistamine spots. After cooling each band was scraped into a 7-ml scintillation vial (FisherBrand™, Fisher Scientific, Houston, TX) followed by the immediate addition of 1 ml EtOH. When all samples had been scraped into vials with the ethanol, 5 ml of ScintiSafe™ Econo F scintillation cocktail was added to each vial. The samples were allowed to sit a few minutes before being placed in a Beckman Scintillation Counter (Beckman Coulter, Fullerton, CA). Five-min counts for ^{3}H were performed on each sample. After subtraction of the mean counts per minute (CPM) value for the blanks, sample histamine levels were calculated using the standard curve plot of CPM against the internal histamine standards run with each assay.

Evan's Blue Determination. Extraction of Evan's blue from tissue using trichloroacetic acid (TCA) was first described by Rossner and Tempel, in 1966 (Rossner and Tempel 1966). The procedures used here for extraction and quantification of Evan's blue were generally based on that described by Uyama, et al, 1988 (Uyama, et al. 1988), although considerable modifications were required. Cortical tissue samples were homogenized for 30 s in 125 µl 50% TCA (w/v) using the Kontes micro-ultrasonic cell disrupter. Homogenized samples were centrifuged at 6,000 g and 4° C for 20 min in a Sorvall ultracentrifuge. 125 µl of the supernatant containing the Evan's blue was removed and diluted with 375 µl 100% ethanol. This formed a 1:3 ratio of the TCA:ethanol solvent mixture. Addition of the ethanol reportedly stablizes the Evan's blue fluorescence which degrades rapidly during measurement. It also stabilizes the Evan's blue for storage, if required, up to about 7 days (Uyama, et al. 1988). Aliquots of the

extracted samples were used to determine quantitative values for the Evan's blue content of the tissues based on a standard curve for Evan's blue. Serum concentrations of Evan's blue was determined using the same procedure except that the aliquot used for the fluorescence reading was diluted 1:20 with the solvent combination. This was required to obtain readings that fell within the standard curve. The numerical value obtained for the serum Evan's blue value was not used in calculations but as a gauge to verify that each mouse received the same amount of Evan's blue.

Evan's Blue Quantitation. These experiments dictated considerable adaptation of the previously published procedures to permit working with much smaller samples of tissue as well as Evan's blue. To accommodate the small sample volumes fluorometer micro cells (Starna Cells, Inc., Atascadero, CA) were used that permitted measurement with sample volumes as small as 315 µl. A special adapter accurately positioned the micro cell in the center of the cell compartment of the spectrophotometer. Fluorescence was measured with an AMINCO-Bowman Series 2 (AB2) Luminescence Spectrophotometer. The spectrophotometer was PC-controlled using AB2 software, version 95.12 (Spectronic Instruments, Rochester, NY), on an OS2 platform. The settings were: excitation wavelength 620 nm with a bandpass of 8 nm, emission wavelength 660 nm with a bandpass of 16 nm, and an instrument offset, determined by a blank sample of the TCA-ethanol solvent. Use of the micro-cells required an increase in the voltage sensitivity from that used for standard sized cells from 605 V to 740 V. The volume of sample used for fluorescence measurements was 320 µl.

Standard samples of Evan's blue (100, 200, 300, 400, and 500 ng/ml) in 50%: TCA 100% EtOH (1:3) were prepared and run with each set of experimental sample determinations. The fluorescence curve for Evan's blue within this range of concentrations has been demonstrated to be linear (Saria and Lundberg 1983, Shapira, et al. 1993, Uyama, et al. 1988).

Zolantidine Effect Experiments. Zolantidine dimaleate (kindly supplied by Leslie Hough, Albany, NY) was administered to the mice in doses of 2.5 mg/kg, 10 mg/kg, or 20 mg/kg in normal saline, 0.2 ml, subcutaneously (s.c.). The doses given are for the zolantidine, not the dimaleate salt. Maleic acid in saline solution was used as the control vehicle injection. Drug or vehicle injections were given immediately after the Evan's blue had been injected. A 30 min wait followed the zolantidine/vehicle administration before the lines were scored in the skull. The mice were kept under anesthesia for a 20 min survival time before perfusion. The drug effects were evaluated by Evan's blue quantitation. Sketches were made of the cortical surfaces. Control animals received 0.2 ml of the malic acid in saline vehicle and the same procedures as experimental mice. Photographs of some of the dorsal cortical surfaces were made using a Nikon digital camera with a Nikon Stereo Photo SMZ-10 Stereoscope (Nikon, Japan). The magnification value for these photos was 25X.

Statistical analysis. All data shown are expressed as mean ± S.E.M. of a group of at least four experiments. Statistical analysis used Student's *t* – Test: Paired Two-Sample for Means, one-tailed. Mast cell counts are presented as a percentage of

degranulated/activated mast cells or non-degranulated/non-activated mast cells per total mast cells counted for the left and the right duras separately. The size of the dural preparation was not identical for all duras but all included the dura subjacent to the traumatized left parietal skull.

CHAPTER 4

RESULTS

Mast Cell Degranulation. To evaluate the time course and extent of mast cell (MC) degranulation (MCD), the surgical procedures were performed on two groups of mice: Group 1: 0 time survival (n = 6), and Group 2: 20 minute survival (n = 6). To simplify distinction of differences only a single line was scored in the left dorsal parietal skull of these animals. This was because often the scoring of two lines produced a wider area of degranulation, which would make it more difficult to obtain clear-cut results.

Using a 1 mm^2 ocular grid (Lee, et al. 1993), all MCs for left and right sides of the dura from each animal were counted. A standard sample count region was not used because there was some variability in placement of the lines on the skull from mouse to mouse. The process of dissecting the duras away from the skulls also sometimes encountered errant cuts, tears, and breaks which resulted in a degree of non-uniformity of dura area. MCs were counted as normal if the staining was dark blue-purple, the cell membrane appeared even and intact and at higher magnifications granules could be seen inside the cell. Unactivated MCs also had a compact appearance (Fig 3(A), (B), and (C)). MCs were counted as degranulated if they were completely degranulated, resulting in a pink color, swollen in size compared to the normal MCs (Fig. 3(E), (G), and (H)). Some

MCs revealed granules bulging from the cell membrane surface and/or releasing contents (Fig. 3(F). For some MCs, the cell membrane would appear disrupted and free granules nearby (Fig. 3(E). MCs completely degranulated appeared very faint and difficult to see

TABLE 1
Percentage dural mast cell degranulation at 0 and 20 min following the experimental trauma.

In this set of experiments only a single line was scored in the left dorsal parietal skull. The duras were fixed in 10% NBF and stained with Toluidine blue (0.5%) in distilled water. The degranulated mast cells in the right and left dura of each mouse were counted. (A) Group 1 mice (n=6) were sacrificed immediately following the trauma (t = 0). (B) Group 2 mice (n=6) survived 20 min after the trauma, under anesthesia, before being sacrificed. Total MCs were counted for the left, and then the right, dura from each mouse. Counts were expressed as percentages total count for each respective dural half.

A.

Mean Percentage Mast Cell Counts
0 min Post-Trauma Time

	Right	Left
Degranulation	2	39
No degranulation	98	61
S.E.M.	0.002	0.013
n =	6	6
p <	0.0000	0.0002

B.

Mean Percentage Mast Cell Counts
20 min Post-Trauma Time

	Right	Left
Degranulation	34	55
No degranulation	66	45
S.E.M.	0.02	0.019
n =	6	6
p <	0.0002	0.0292

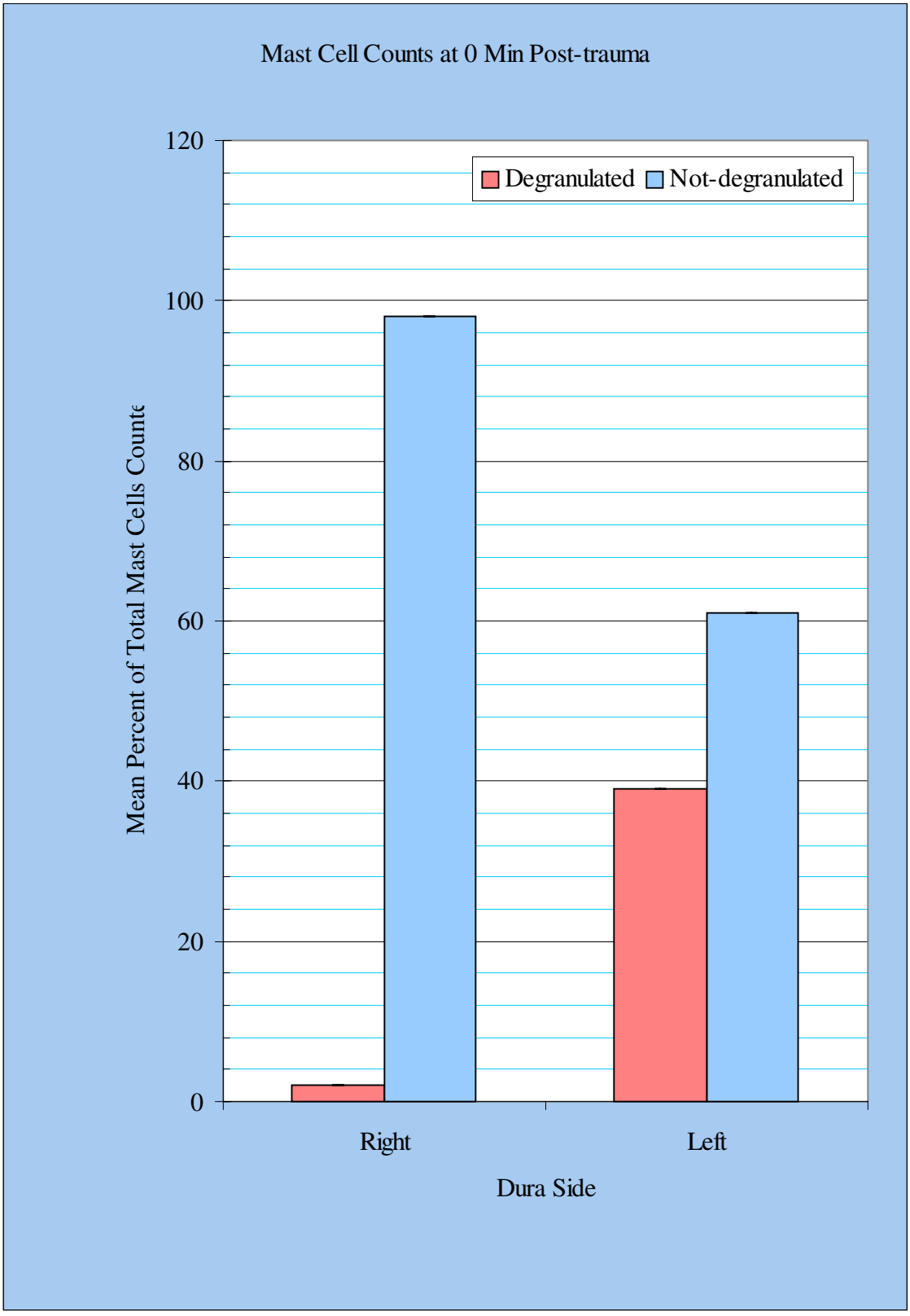
Mast Cell Counts at 0 Min Post-trauma
Degranulated
Not-degranulated
120
100
80
60
40
20
0
Mean Percent of Total Mast Cells Counte
Right
Left
Dura Side

Fig. 2A. Mast cell (MC) counts in right (untraumatized) and left (traumatized) duras. Chart shows mean percent of degranulated/activated versus the normal/unactivated MC at (A) 0 min survival time. Data expressed as Mean ± S.E.M.

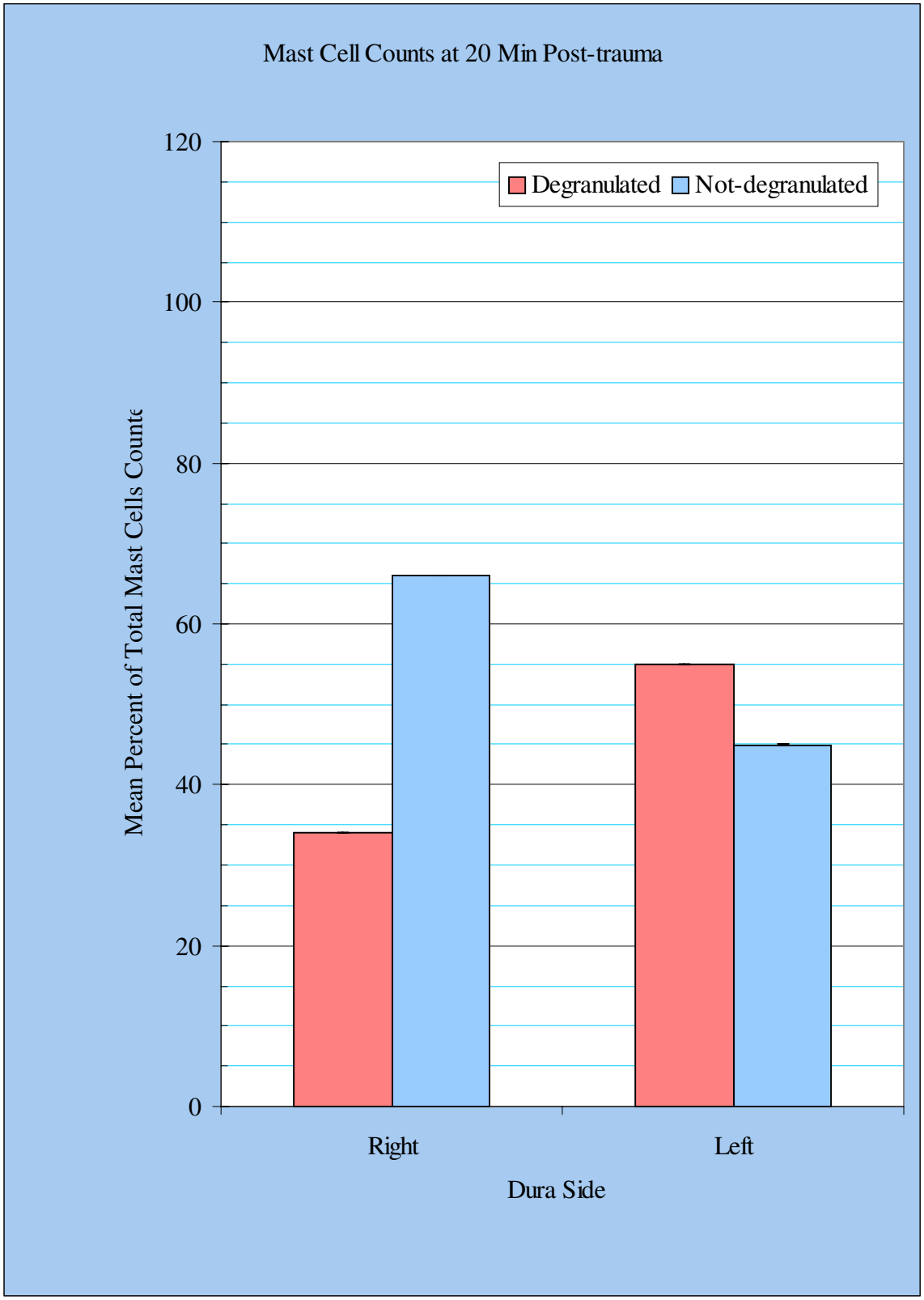
Mast Cell Counts at 20 Min Post-trauma
Degranulated
Not-degranulated
120
100
80
60
40
20
0
Mean Percent of Total Mast Cells Counte
Right
Left
Dura Side

Fig. 2B. Mast cell (MC) counts in right (untraumatized) and left (traumatized) duras. Chart shows mean percent of degranulated/activated cells at (B) 20 min survival time, following the experimental head injury. Data expressed as Mean ± S.E.M.

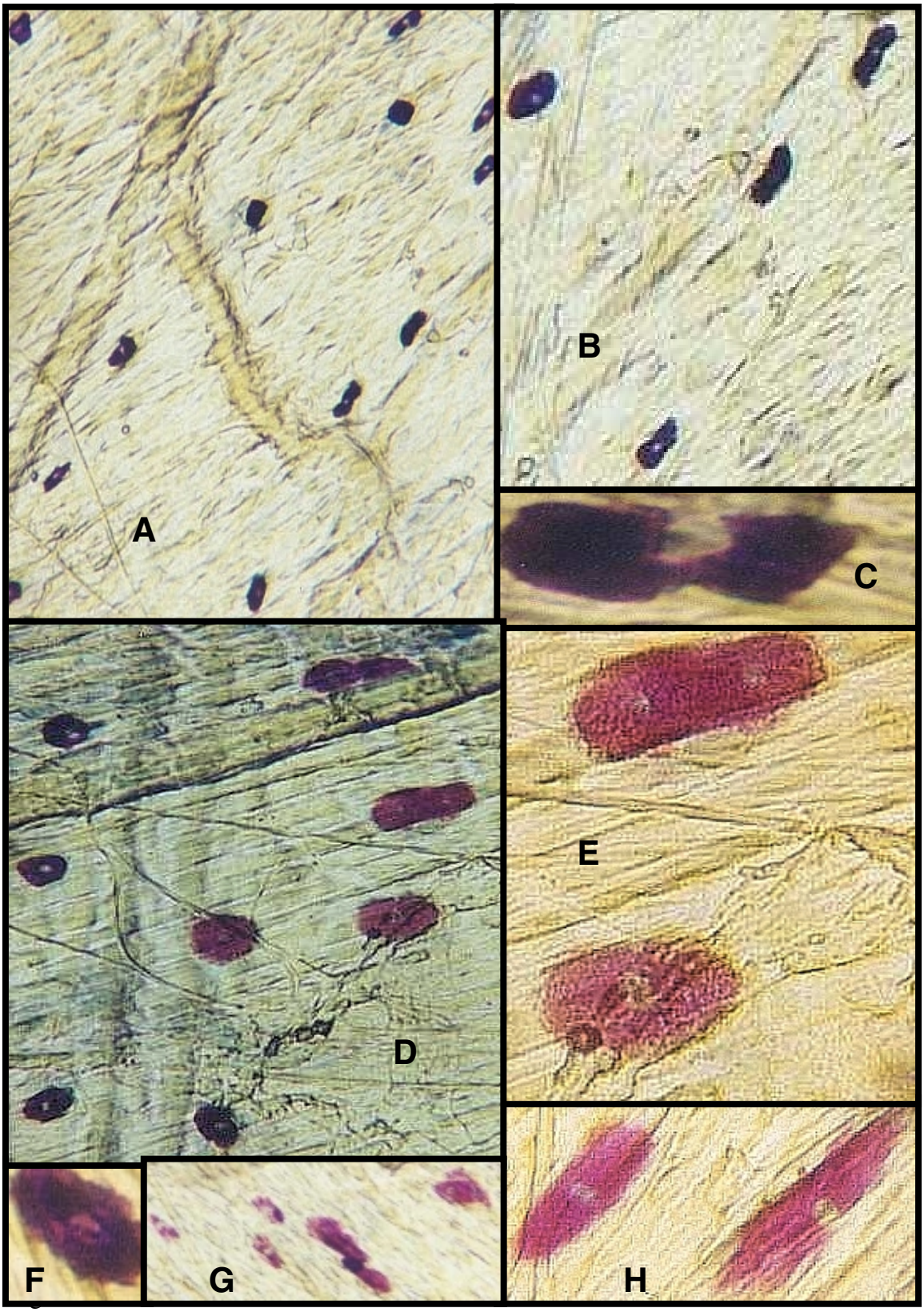
A
B
C
D
E
F
G
H

Fig. 3. Photomicrographs of dural mast cells (all photographs on this page made from a single slide): (A) normal (40X), (B) normal (100X), (C) normal MC (240X). (D) normal MCs in the same field as degranulated MCs. Normal MCs are to the *left* of the middle meningeal artery and degranulated MCs are to the *right.*(100X), (E) degranulated MCs (200X), (F) an activated and degranulating MC as indicated by the uneven cell membrane, the swollen cell volume, and the metachromasia of the weakened stain. At a lower magnification this cell might be mistakenly counted as a normal MC, (G) a group of degranulated MCs in the dura (40X), (H) degranulated MCs (160X). (Zeiss phase contrast with Olympus PM-6 camera back and Kodak Gold 100 35 mm film).

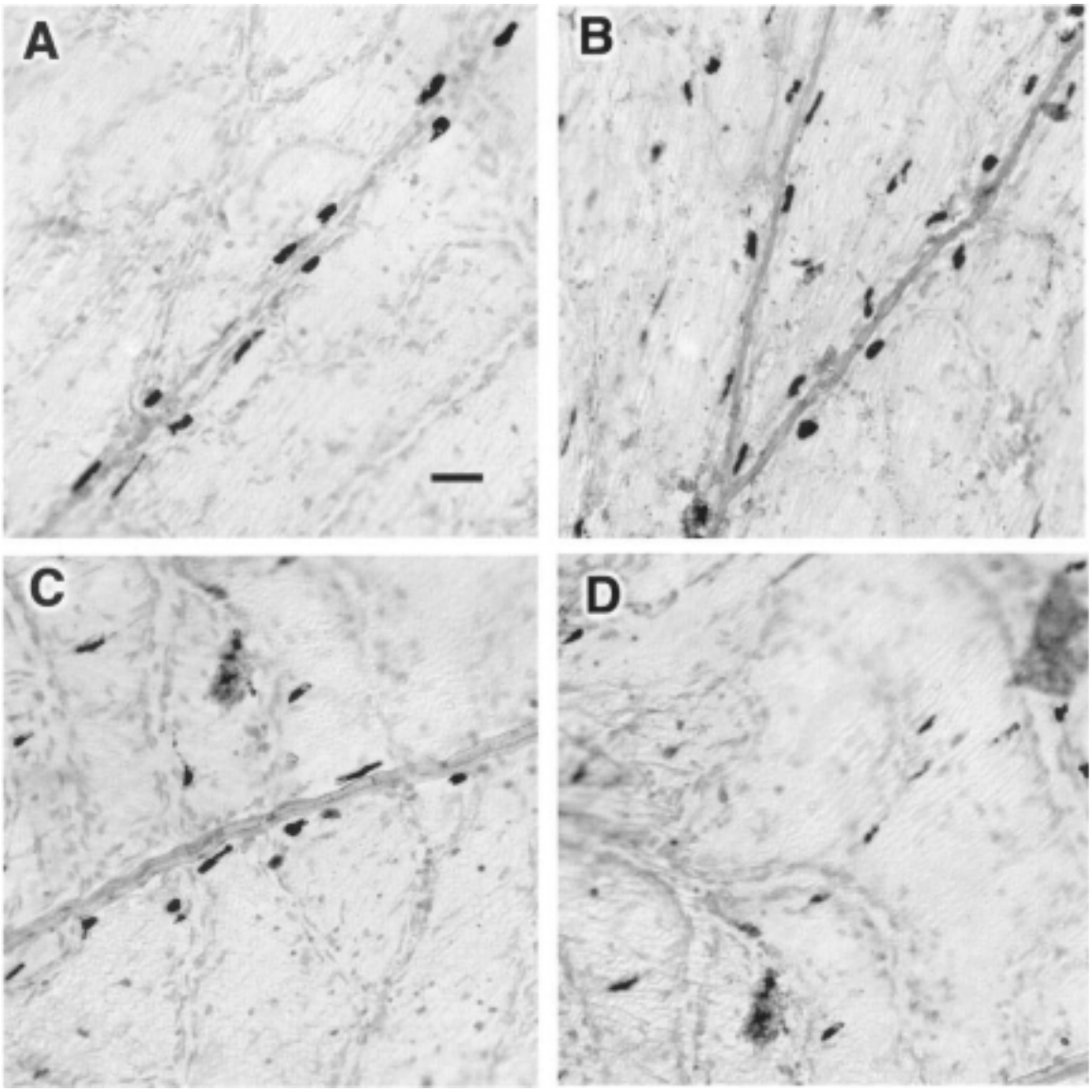
A
B
C
D

Fig. 4. (A) Normal MCs aligned along artery in dura, (B) and (C) show normal and degranulated or activated MCs, and (D) shows mostly degranulated MCs. Bar represents 55 μm.(Nikon Microphot FXA)

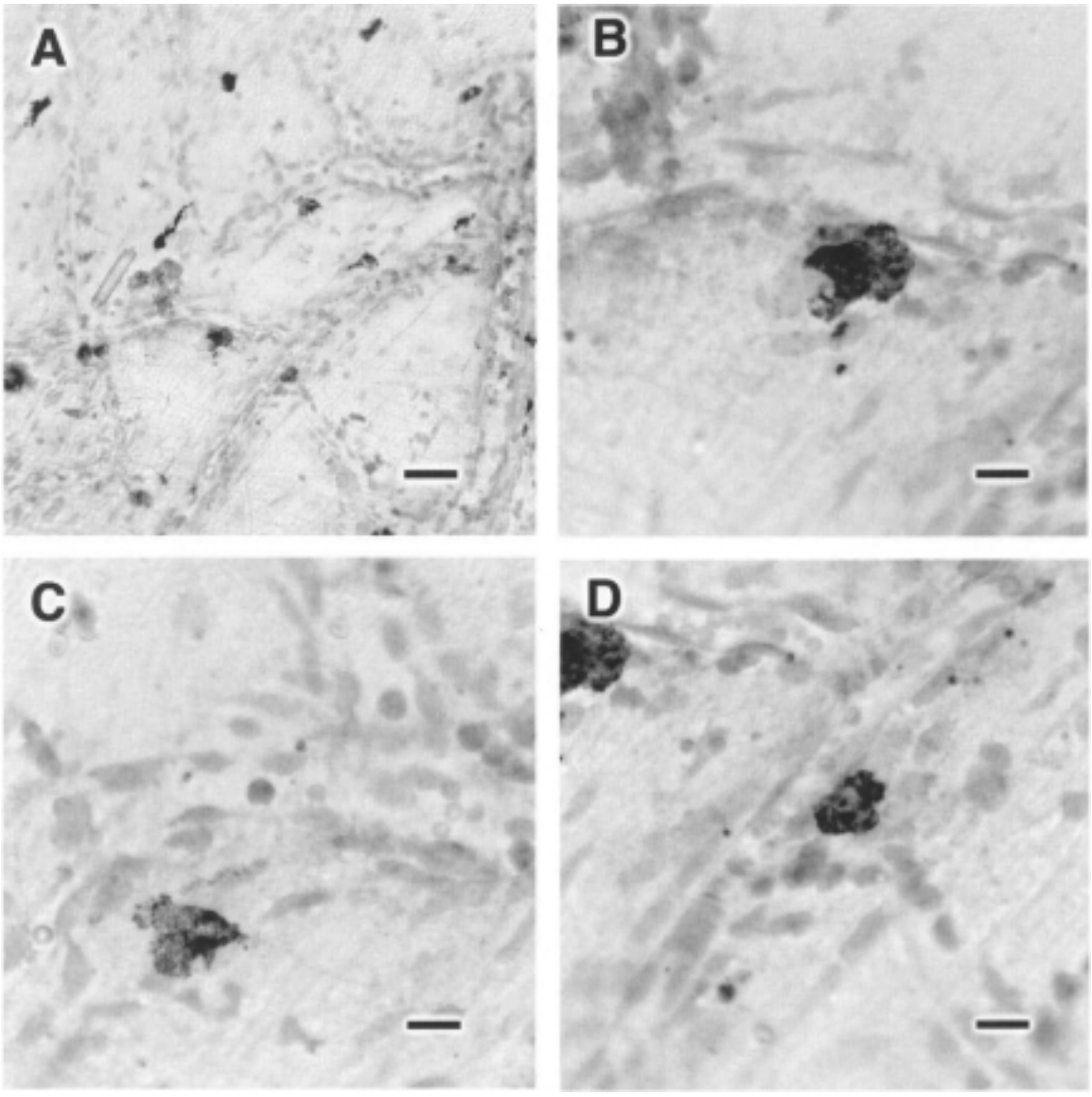
A
B
C
D

Fig. 5. (A) MCs degranulating, (B), (C), and (D) are enlargements of cells from A Bar in A = 55 μm; bar in B, C, and D represents 14 μm. (Nikon Microphot FXA)

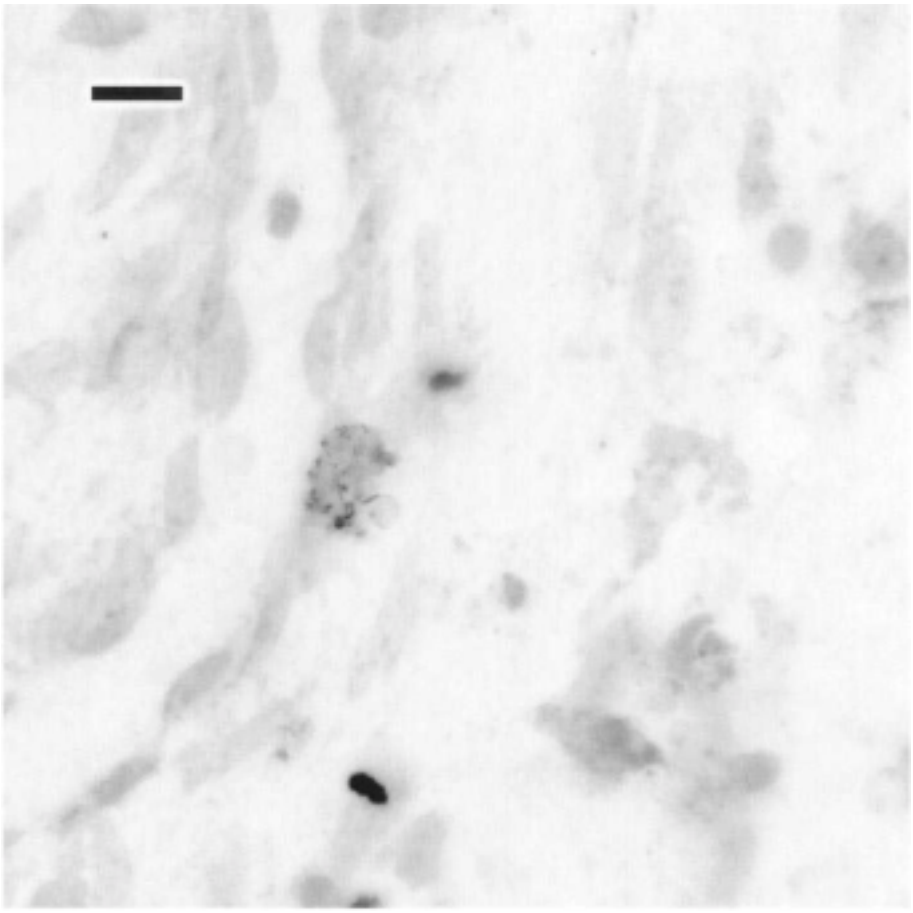

Fig. 6. MC in center was massively degranulated. Viewed with the Zeiss microscope, it was visible as a faint cluster of light pink dots. Bar = 14 μm.(Nikon Microphot FXA)

(Fig. 6): like an aggregation of pink granules without a membrane. This lack of visual clarity was compounded when the MC was a little deeper in the connective tissue than more superficial MCs in the focal plane. Determination of MC activation/inactivation state was based on the descriptions and photographs from the literature (Berlin and Enerbach 1983, Berlin and Enerbach 1984, Bloom and Chakravarty 1970, Bloom, et al. 1967, Bloom and Haegermark 1965, Bytzer, et al. 1981, Enerbach 1974, Lagunoff 1972a, Lagunoff 1972b, Lagunoff 1973, Nielsen, et al. 1981, Thon and Uvnas 1967).

Each dura demonstrated a full range of partially activated to fully degranulated cells. Typically, the area subjacent to the traumatized area of the skull had the densest concentration and grouping of fully degranulated cells. More to the periphery here was an increase in the number of cells activated and partially degranulated. Going further out, degranulated and activated MCs appeared in the same fields as normal MCs (Fig. 3(D)). At the most distant portions of the dura, groups of normal-looking mast cells were frequently observed.

Immediately after the trauma, the right dura had only 2% of its mast cells visibly activated or degranulated ($n = 6$, $p < 0.001$), whereas for the left dura this was 39% ($n = 6$, $p < 0.001$). At 20 min following the trauma, the right side demonstrated 34% activated or degranulated mast cells ($p < 0.001$) and the left dura showed a further increase to 55% ($p < 0.05$).

There was also a small increase in number of degranulated/activated MCs on the right untraumatized side (Fig. 4(D)).

Histamine Determinations in Cortex and Dura. The left cortex subjacent to the trauma showed increases in histamine compared to histamine levels measured in corresponding areas of the right cortex. At 5 min there was an increase of 154% histamine on the left ($n = 7$, $p < 0.01$), at 10 min, the increase was 174% ($n = 6$, $p < 0.02$), and at 20 min the increase was 151% ($n = 13$, $p < 0.01$) (Table 2. (A); Fig. 7. (A)). Simultaneously there were decreases in histamine content of the corresponding duras as measured at time points of 5 and 10 min following the trauma. At 5 min histamine in the left dura was 59% compared to the right dura ($n = 7$, $p < 0.05$), and at 10 min this was 75% that of the right dura ($n = 5$, $p < 0.05$) (Table 2. (B), Fig. 7. (B)).

BBB Permeability to Evan's Blue. Quantitation of Evan's blue extravasation into the cortical tissue was measured at 20 min post trauma. The left cortex demonstrated an increase of 1385% over the value measured for the right cortex ($n = 9$, $p < 0.001$) (Table 3, Fig. 8). Typically, the fluorescence determinations for samples from the right (control) hemispheres were elevated above that of the blank (solvent mixture) value, compared to the values from cortical samples from sham procedure mice (no Evan's blue) (data not shown).

Zolantidine Study. Zolantidine, a potent and selective histamine H_2 receptor antagonist, is also permeable to the blood-brain barrier, which makes it unique among H_2 antagonists. Zolantidine was administered 30 min before the surgical trauma to attempt to block the H_2 receptors in the blood-brain barrier and determine to what degree, if any, it could moderate the histamine-mediated increase in permeability following the trauma.

The control solution, maleic acid in saline, was prepared according to the maleic acid concentration of the drug solution. The maleic acid component of the zolantidine dimaleate constitutes 50% of the formula weight of the compound. Zolantidine administered at 2.5 mg/kg (n = 3) demonstrated no ability to reduce the Evan's blue extravasation. The resultant values were not significantly different (p = 0.436) from those of the control animals (n = 3). Zolantidine at the 10 mg/kg dose (n = 4), and at the 20 mg/kg dose (n = 3), were associated with a considerable reduction in Evan's blue extravasation, suggesting a blocking effect for some of histamine's effects. Because there was also no statistical difference between data from the 10 and 20 mg/kg dose groups (p = 0.187), for statistical evaluation, the data from the control animals and the 2.5 mg/kg group were pooled (n = 6). Likewise the data from the 10 mg/kg group and the 20 mg/kg group, were also pooled (n = 7) (Table 5). The analysis indicated that giving the 10 or 20 mg/kg of zolantidine 30 min before the trauma resulted in a 64.56% reduction in Evan's blue extravasation compared to the control plus 2.5 mg/kg pooled group ($p < 0.001$) (Fig.. 9). Photographs of cortical surfaces from two mice are shown in Fig. 11. Photograph 11. (A) is the brain from a maleic acid/saline-treated mouse. Photograph 11. (B) is from a mouse treated with 10 mg/kg zolantidine.

Table 2. (A) Cortical histamine (pg/mg tissue wet wt) at survival times of 5, 10, and 20 min post trauma. (B) Dural histamine (pg/mg tissue wet wt) levels at survival times of 5 and 10 min post-trauma.

Table 2

A. Change in Cortical Histamine Post-trauma

	5-Min	10-Min	20-Min
% Increase	154	173	151
S.E.M.	16	24	15
n =	7	6	13
p <	0.009	0.018	0.006

B. Change in Dural Histamine Post-trauma

	5-Min	10-Min
% Decrease	59	76
S.E.M.	10	9
n =	7	5
p <	0.021	0.021

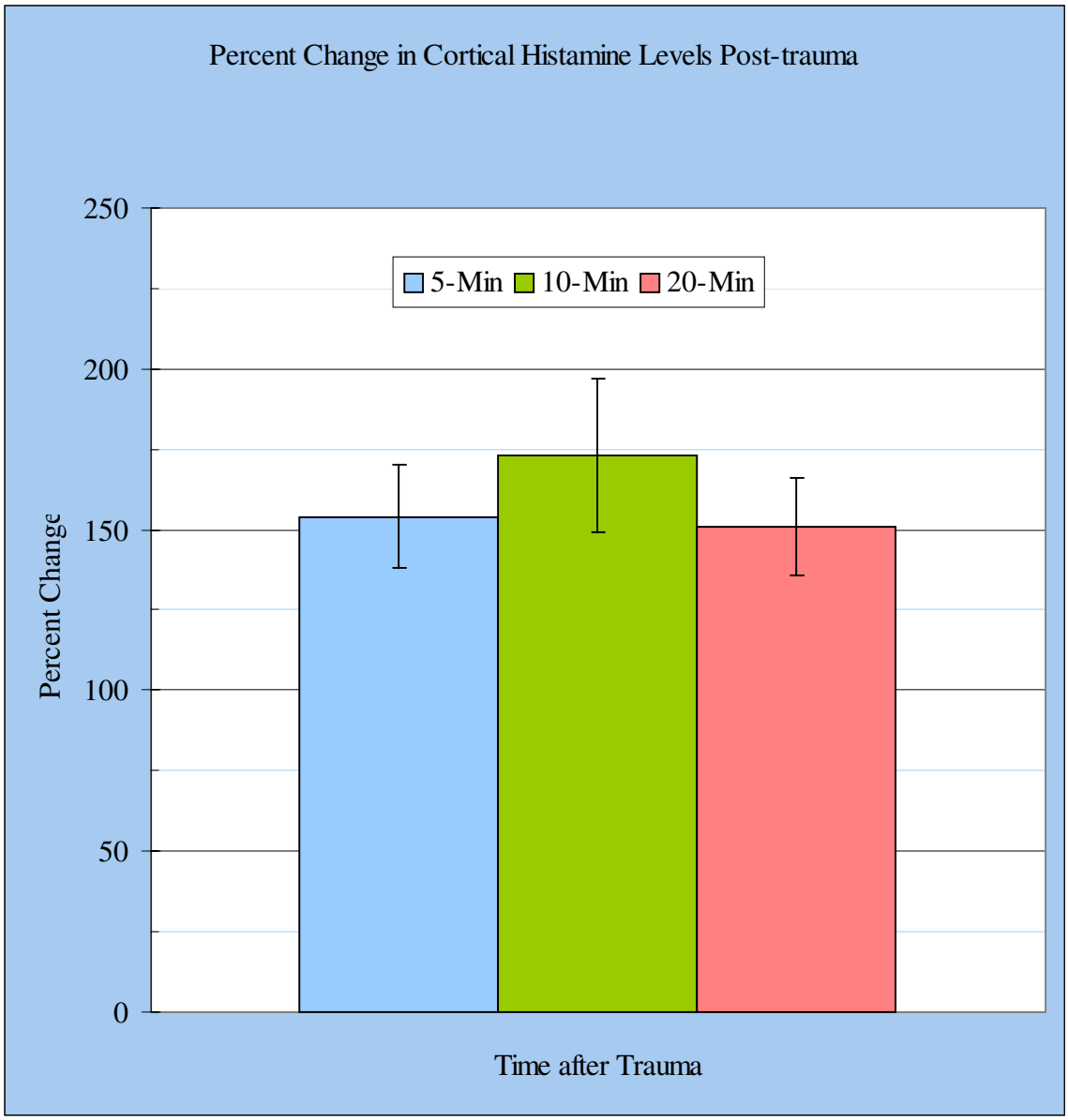
Percent Change in Cortical Histamine Levels Post-trauma
250
200
150
100
50
0
Percent Change
5-Min
10-Min
20-Min
Time after Trauma

Fig. 7A

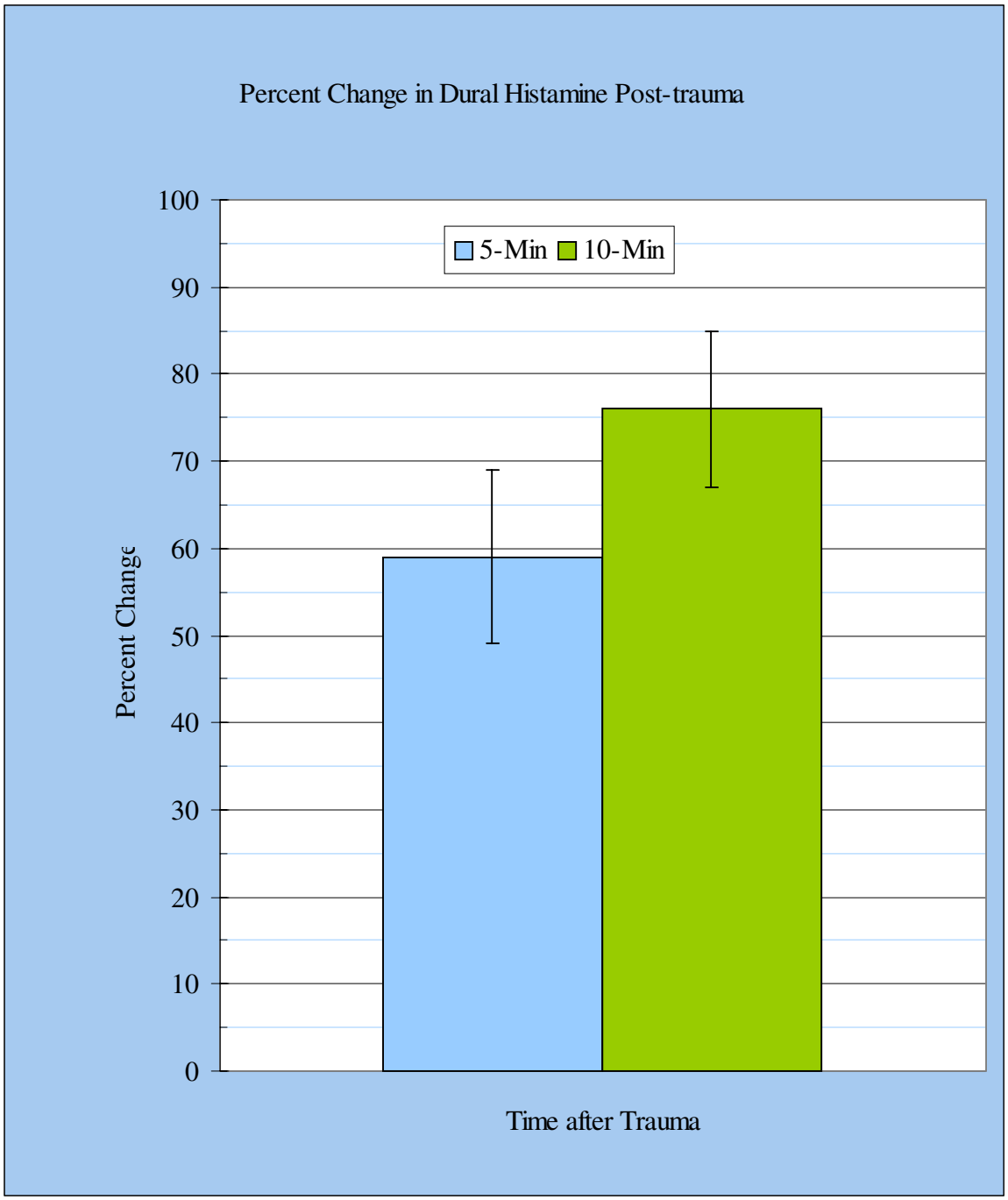
Percent Change in Dural Histamine Post-trauma
100
90
80
70
60
50
40
30
20
10
0
5-Min
10-Min
Percent Change
Time after Trauma

Fig 7 B

Fig. 7. (A) Increased cortical histamine levels (pg/mg) at survival times of 5, 10, and 20 min (B) Decreased dural histamine levels (pg/mg) at survival times of 5, and 10 min. A radioenzymatic histamine assay was used to determine histamine levels. Data expressed as mean ± S.E.M. Student's *t*-test: paired two sample for means, one-tailed.

Table 3.

Cortical Evan’s blue extravasation at 20 min post trauma.

Two cortical plug tissue samples were taken from left and right cortices of the mice (n = 9). Mean concentrations of Evan's blue (ng/mg tissue wt.) were determined using a quantitative spectrofluorometric assay. The results were expressed as mean ± S.E.M. Student's t-test: Paired two sample for means, one tailed, $p < 0.000$.

Table 3.

Cortical Evan's Blue 20-Min Post Trauma (ng/mg)

Cortex	Right	Left
Mean	175	2424
S.E.M.	34	392
n =	9	9
$p <$	0.000	

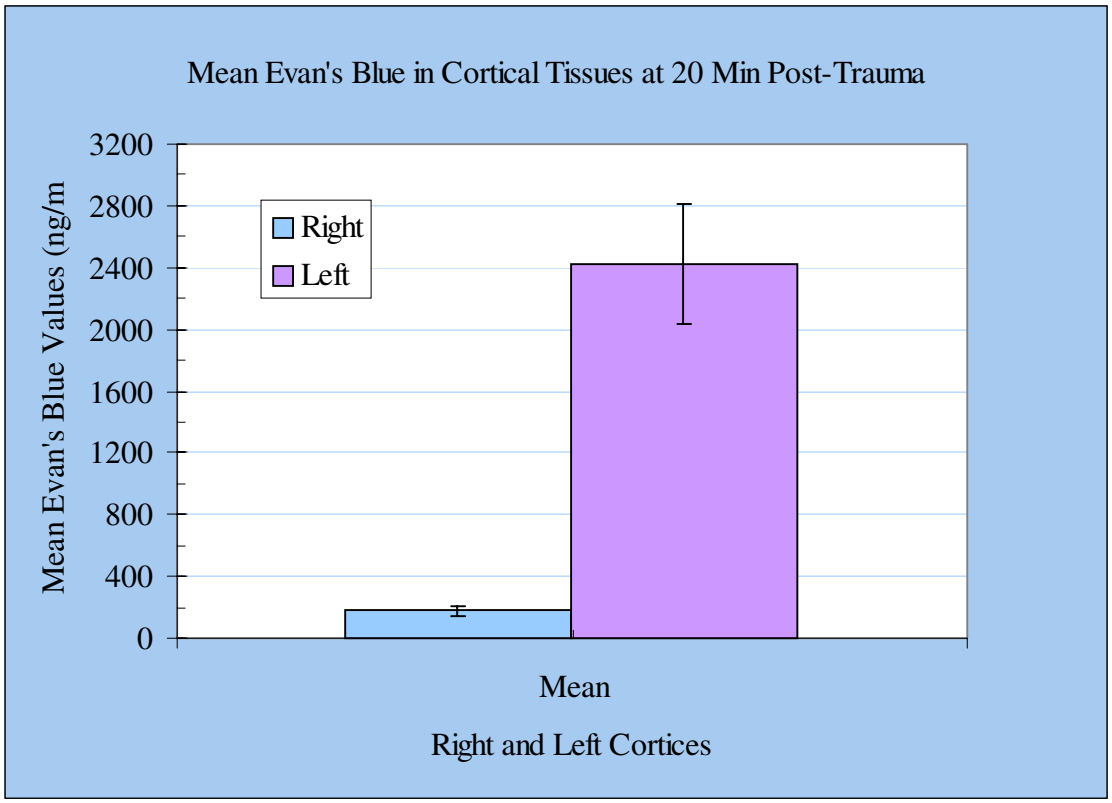

Fig. 8

Fig. 8. Mean Evan's blue concentrations (ng/mg) in the traumatized left (n = 9) versus the untraumatized right (n = 9) cortex at 20 min survival time ($p < 0.001$). Chart shows the mean Evan's blue concentration ± S.E.M. Student's t-test: paired two sample for means, one-tailed.

Table 4.
Effect of zolantidine on Evan's blue extravasation 20 min post trauma.

Control mice received injection of 0.2 ml maleic acid (ma) in saline, s.c., 30 min before the two lines were scored in the skull. The experimental mice received 2.5, 10, or 20 mg/kg zolantidine, 0.2 ml injection, s.c., also 30 min before the line scoring. The mice remained anesthetized for the duration of the experiment. Data expressed as mean ± S.E.M.

Table 4 Effect of Zolantidine Dosage on Evan's Blue Extravasation

	maleic acid	2.5 mg	10 mg	20 mg
Mean	1566	1532	561	534
S.E.M.	253	69	97	97
n =	3	3	4	3

Table 5.
Data summary for zolantidine effect on Evan's blue extravasation.

For purposes of statistical analysis data from control mice and 2.5 mg/kg mice were pooled and data from 10- and 20 mg mice were pooled. Data from the two pooled groups was expressed as mean ± S.E.M.

Table 5 Zolantidine Effect: Summary data

Left Cortical Evan's Blue Levels (ng/mg)

	ma+2.5 mg	10+20 mg	Change
Mean	1549	548	65%
S.E.M.	17	14	
n =	6	7	
p <	0.001		

(ma = maleic acid in saline control)

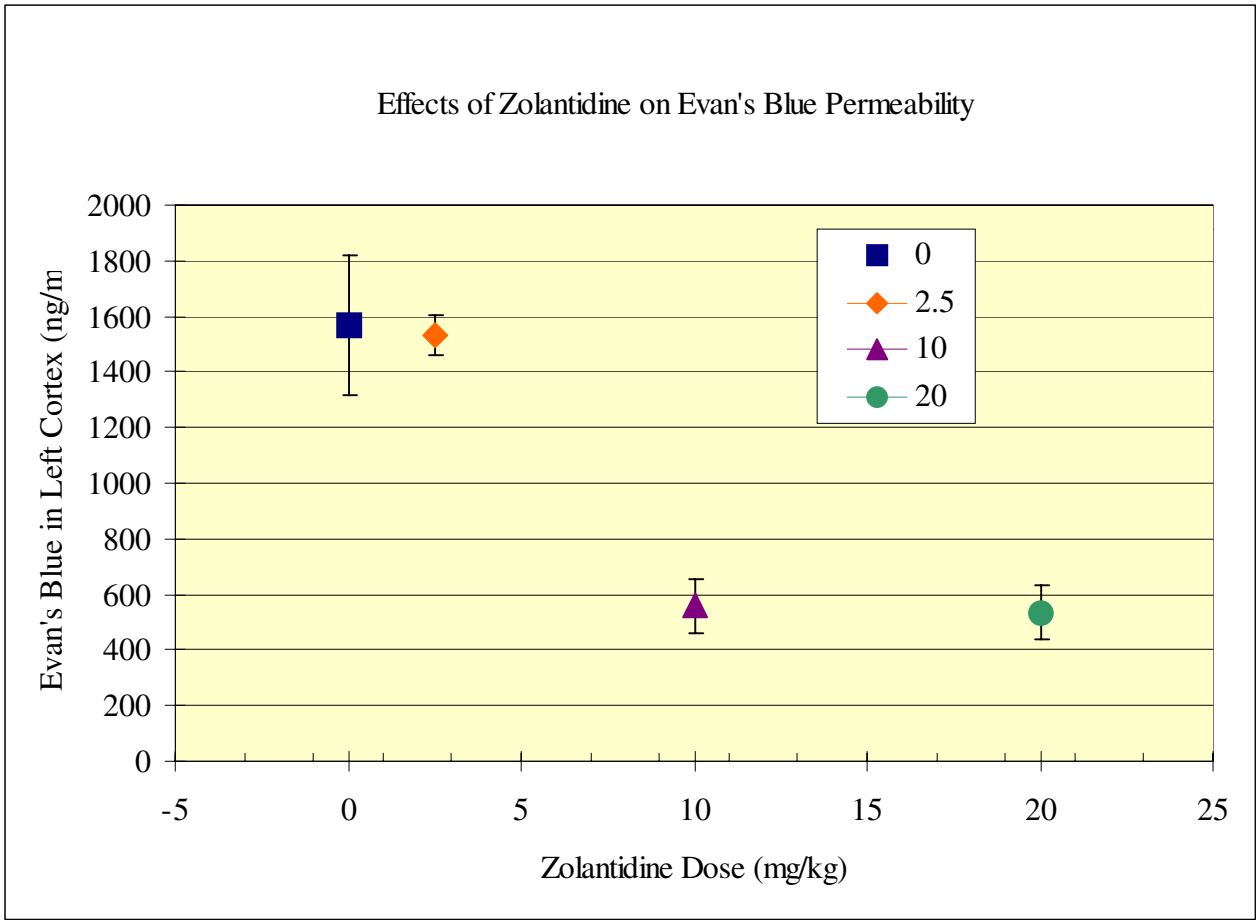

Fig. 9

Fig. 9. Zolantidine suppression of Evan's blue extravasation at post trauma survival time of 20 min. Scatter plot depicts the Evan's blue measured in the left cortex (ng/mg) less that in the right cortex. The mean ± S.E.M. Evan's blue values for each dosage group are plotted: control (n = 3), 2.5 mg (n = 3), 10 mg (n = 4), and 20 mg (n = 3).

Table 6.
Evan's blue standards accompanying the zolantidine experiments.

The standards were assayed the same afternoon as the tissue samples from the zolantidine experiments.

Table 6 Evan's Blue Standards
Zolantidine Experiments

Evan's Blue (ng/ml)	Fluorescence Units
0	0.000
100	0.410
200	0.800
300	1.212
400	1.655
500	2.098

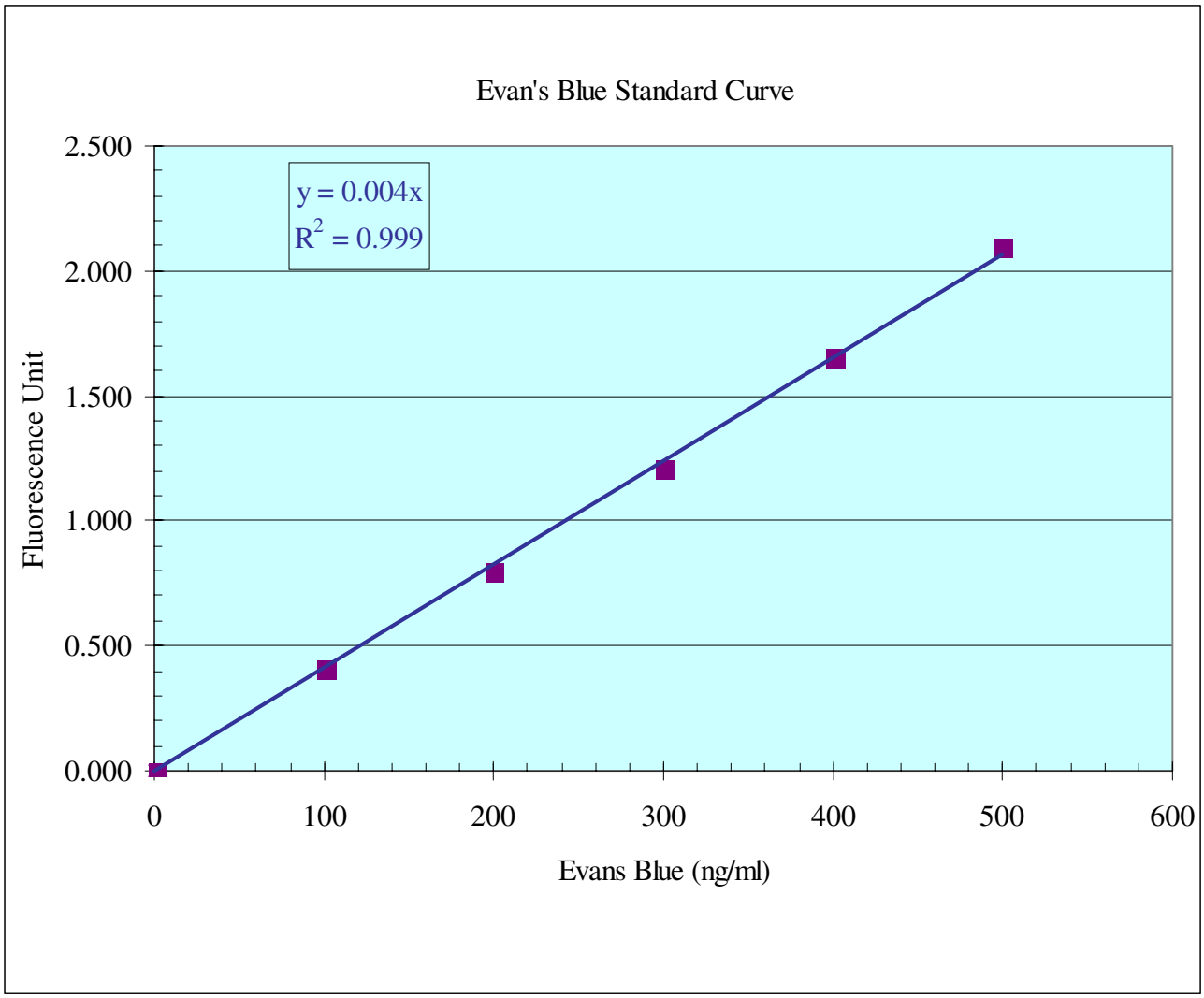

Fig. 10.

Scatter plot standard curve of Evan's blue standards of 100 - 500 ng/ml Evan's blue.

These standards were assayed with tissue extracts from the Zolantidine experiments mice.

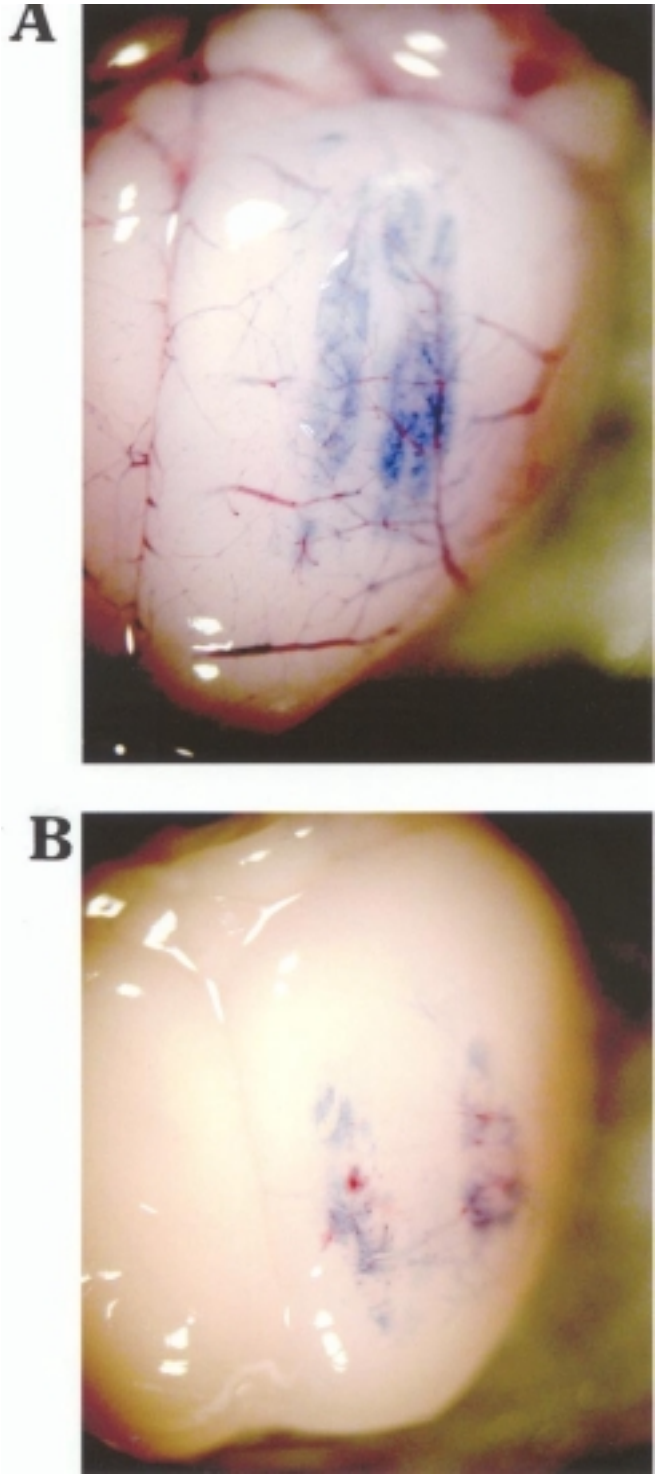
A
B

Fig. 11. Photographs from zolantidine experiments. (A) The cortical surface from mouse pretreated with the maleic acid control. The Evan's blue extravasation is evident on the left dorsal parietal cortex. The right hemisphere has no visible bluing. (B) The cortical surface from a mouse pretreated with 10 mg/kg zolantidine. Visual comparison with the photo from the control treated mouse shows a distinct reduction in the intensity of extravasated Evan's blue. Cortical plug samples were made from the blue areas of the left hemispheres. Control cortical samples were taken from corresponding areas of the right hemisphere. (25X) (Nikon digital camera)

CHAPTER 5

DISCUSSION

Traumatic Brain Injury Model. The literature on TBI is almost entirely of studies on severe TBI. Over the last ten years, more studies have appeared on MTBI. The more severe the models of injury, the more extensive the damage and the more difficult to isolate the damage processes. The model of MTBI used in these experiments was developed in this lab. Cutting the lines halfway through the skull appeared to be a useful model although it lacked the more standard features of other models. The model does have some limitations. One is a degree of variability. However the skull of the mouse is thin and for the mice used in this study, essentially of uniform thickness, about 1 mm, as well as width and length. This controls, to a considerable degree, how variable the trauma-inducing event can be. It has to produce an effect without cutting completely through the skull and dura. Often the more variable components were positioning of the scored lines and the width and thickness of the lines. Usually the cut for a line was repeated 2-3 times and almost never did the lines fall exactly in the same precise groove. Additional factors that must be mentioned because they are not components of the fluid percussion or cortical impact type of trauma models. These are heat and vibration. The cutting wheel does generate some heat when used. Because the cutting process takes only

about 15 s and the wheel is not held in a single spot the entire time, the heat effect may be minimal. It may be contributing some injury effect, this is not known. The Dremel tool and cutting blade transfer a small but definite vibration force when in contact with the skull. This may contribute to a mechanical distortion of mast cell membranes triggering a depolarization with calcium entry and degranulation. The vibration plus the force of the cutting wheel pressed on the small skull may be the main triggers of degranulation by acting at mechanosensitive or stretch activated ion channels in the mast cell membrane.

Mast cell degranulation. Initial observation of stained dural mast cells following the experimental trauma suggested that the number of clearly degranulated mast cells immediately after the trauma appeared in an area of the dura underlying the lines scored into the skull. With the survival time increased to 20 min, the area of degranulation seemed more extensive. There also seemed to be more degranulated mast cells on the untraumatized right side. This was also of interest because the histamine levels measured in the cortex were elevated to about the same percentage of normal whether measured at 5, 10, or 20 minutes. If the initial impact was the only triggering factor for the degranulation, then it would be more likely to expect a peak histamine release early with a rapid decline.

Although the increases in histamine content of the left cortical areas were vastly less elevated than the corresponding decrease in histamine content from the corresponding duras, the increase was significant. The observation that the level remained elevated at essentially the same level for at least the first 20 minutes after the trauma indicated that

there might be a continued release of histamine, rather than a single large bolus release. Considering the results of the degranulation counts, this data seems to indicate there is a spreading area of MC activation contributing to a sustained elevation of free histamine. The appearance of increased numbers of degranulated or activated MCs on the right cortex may help explain why there is a slightly elevated Evan's blue level measured from the right cortex compared to the blank. It is also in agreement with several other studies that have reported the development of small increases in BBB permeability in the contralateral cortex and hippocampus. While this is observed, it has not been shown as associated with subsequent edema formation or other pathological after effects of the trauma event (Kapasi and Povlishock 1991, Shapira, et al. 1993).

It was observed in this study, consistent with other reports in the literature, that the distribution of MCs is not uniform in the dura mater. The densest populations of mast cells were seen in the areas closest to the middle meningeal arteries (MMAs) and the central saggital sinus. MC numbers progressively decreased to the more peripheral areas. The MMAs constitute the primary arterial supply to the duras. The position of the MMAs in the dura essentially overlies the position of the middle cerebral arteries (MCAs), the major arterial supply to the cerebral hemispheres. This may help explain why direct damage to the dorsal parietal area of the brain, in both mice and humans, results in so much permanent neurological impairment of functions related to these areas. Additionally, many of the studies on brain edema and BBB breakdown have used experimental models of occlusive stroke. Several of these studies have also tested H_1 and

H_2 antagonists for therapeutic potential at the BBB. Much of the literature supportive of application of H_2 antagonists to ameliorate increased permeability of the BBB comes from these studies. The most common type of stroke is one that involves a middle cerebral artery (MCA) and this is a common animal model in which to study stroke (Belayev, et al. 1996, Cole, et al. 1990, Cole, et al. 1991, Kuroiwa, et al. 1985). The MCAs are positioned almost directly under the dural MMAs. Although it is commonly accepted that the primary edema in stoke is a cellular edema, it also now accepted that the cellular and vasogenic edema are equally important, that the two cannot be distinctly separated. A stroke in an MCA would be more likely to generate mediators able to diffuse to some extent across to the dense population of MCs in the MMA area, triggering degranulation and accelerating BBB permeability changes and edema formation.

Cortical Histamine Levels. Preliminary studies for these experiments used male CF-1 mice. Obtaining quantifiable amounts of cortical histamine from the mice was often difficult. The cortex (and hippocampus, also cortical tissue) has a very sparse distribution of mast cells. There is a basal level of histamine in brain cortical tissue. The source of this histamine is now primarily attributed to histaminergic neuronal release. Histamine, like other biogenic amine neurotransmitters is widely and diffusely projected to the brain and spinal cord from neuron cell groups in the tuberomammillary nucleus (Kandel, et al. 2000, Lin, et al. 1996, Schwartz, et al. 1980). Several factors may have contributed to the low histamine. Some strains of animals have lower or higher populations of dural mast cells. In addition, there can also be species-specific differences in the contents of mediators, both type and amount (Johnson, et al. 1991). The decision to switch to the

SLJ/J mice was based on the knowledge that these have several times as many dural mast cells (Bebo, et al. 1996, Johnson, et al. 1991, Yong, et al. 1994). Another factor could have been that they were male mice housed two to a cage with center partition. Despite the partition, the mice still managed to inflict considerable damage on each other's tails, feet, and ears. It is possible that, although separated, the two male mice certainly were aware of each other's presence and maintained a readiness to fight continuously. This must have created a higher level of chronic stress than a freely living mouse might be expected to experience. This could have affected mast cell activity through feedback from the HPA (Theoharides, et al. 1995). Some of the CF-1 mice had been in the same housing arrangement for well over 6 months when used for experiments.

The measurement of cortical histamine was used as a marker to indicate that histamine released from the dural mast cells had been able to diffuse to, and across, the BBB. The actual changes in dural histamine (in picograms) were very large in comparison to the increases observed in the cortex. Most of the released histamine probably was inactivated by HMT, present both extracellular and in the blood vessel walls of the dura and brain (Hough 1999). There is evidence that brain trauma impairs the ability of HMT to inactivate histamine. However, we do not know to what extent this may have affected the results of these measurements. Some of the histamine was also certainly lost to the cerebrospinal fluid (CSF) as it crossed the arachnoid space. A large amount of the released histamine was likely removed by the circulatory system - blood vessels of the dura, and the pial vessels. Histamine in the circulation does not cross the intact BBB (Hough 1999). Histamine entered the brain cortex after disrupting the integrity of the BBB. Astrocytes have stores of HMT and an uptake mechanism for

histamine. However, traumatic brain injury is well known to cause a metabolic shock to both neurons and astrocytes in the affected area and to disrupt normal cellular function. It is plausible that the major homeostatic functions of the astrocytes were not operational. This places a question over whether or not astrocytes are a major player in histamine removal under trauma conditions. Regardless of the mechanisms involved in removal of the excess histamine, there was a clear increase in histamine in the cortex subjacent to the injury. This increase was significant at 5, 10, and 20 min after the trauma. At 5 min, it was 154% of control, at 10 min, it was 173% of control, and at 20 min, it was 151% of the control value. There appears to be a peak at 10 min. Whether this is important or not is difficult to determine. Since there were only 7 mice for the 5 min group, and only 6 for the 10 min group, it is possible that, with additional repetition and greater numbers, the peak will level out more with the histamine increases measured for the 5 and 20 min groups. In the Mohanty et al. study that used a stab wound to the brain (Mohanty, et al. 1989) the researchers did not see any statistically significant increases in brain or plasma histamine until 5 h after the trauma. This may have been because they were measuring histamine in entire brain hemispheres rather than affected tissues. It is interesting that they also report an increase on the untraumatized side. They found that administration of cimetidine prevented the histamine increase in the brain tissue and the plasma and prevented the edema development. It is not known for certain just how long the increased permeability of the BBB persists, or whether this is something that varies with the type and degree of the trauma. It has been reported that the BBB permeability peaks at around 4-6 hours while the edema doesn't peak until around 24 hours.

Dural Histamine Levels. In attempting to quantify changes in dural histamine content there was an unexpected difficulty. After dissecting the dura from the skull, it had to be transferred into the microcentrifuge tube. In most instances, this process of dissecting and transferring the tissue went smoothly. However, in a number of instances, unexpectedly, the dura would become difficult to detach from the transferring instrument, requiring a little extra time more than usual. Unfortunately, this extra time was enough to allow the dura to dry out much more than the other duras resulting in a very low tissue weight. The low weight of the dehydrated tissue then would throw all the calculations extremely off. There were also some variations between values for individual mice. It was decided to not continue with the dural histamine determinations for the 20-min survival mice. Therefore, the results given are only available for the five and 10-min survival periods. The histamine at 5 min was 59% (n = 7) that of the control side and at 10 min was 76% (n = 5) of the control value. This seems to suggest that at 10 min there was less change in dural histamine content than at 5 min. Even though both results were significant (p<0.05) in both groups, the differences may be an artifact of the low sample numbers. Nevertheless, the results clearly show that the dura on the traumatized left side experienced a significant reduction in histamine content compared to the untraumatized right dura. This is consistent with the previous report (Orr and Stokley 1995) from crainectomy experiments that reported meningeal HA content to be decreased to 62.7% at 10 minutes survival time.

Evan's Blue Extravasation. It was anticipated that there might be some difficulty in quantification of the Evan's blue in the cortical samples from these experiments. Previously published studies all had used larger animals, larger tissue samples, and more

severe models of trauma. They also were working with larger sample aliquots. In addition, the experiments in this study were working with much shorter times following the trauma than previous studies. It was necessary to reduce the volume of the solvents and the sample aliquots to avoid diluting the Evan's blue in our tissue samples below measurable concentrations. A procedural scale-down was required.

It is of interest that we consistently obtained higher fluorescence readings in the right, untraumatized, sample than for the blank. Samples prepared from tissue collected from untraumatized mice who received no Evan's blue injection read the same values as the blank. However, the right cortical values were often several times higher than the blank. The value measured for the left cortical sample was always sharply higher than that for the right, with the exception of the animals receiving no trauma, or no trauma and no dye. Other researchers have observed similar results. Others have observed that there is a small increase in BBB permeability without edema formation (Shapira, et al. 1993). Several possible interpretations were given. One was that their quantitative method for Evan's blue was much more sensitive than the one used to determine edema (water content or specific gravity). It is also possible that extra water does cross the BBB but that it is such a small amount that it is removed via the CSF. It could also be that there does exist a low-grade permeability defect related to release of mediators that is not enough to create edema. Closed head trauma is accompanied by breaking of tiny blood vessels in many areas, including regions distant from the impact (Shapira, et al. 1993). Evan's blue could leak through to the brain from such broken vessels. Another study used antibodies to albumin to visualize the BBB defect specifically (Kapasi and Povlishock 1991).

Zolantidine Effects. It was of interest to use zolantidine as the H_2 antagonist in attempting to block the histamine effect on increasing the permeability of the BBB. Zolantidine is highly selective for the H_2 receptor. Cimetidine, which has been successfully used in a number of studies, can interact with other receptors, somewhat. Cimetidine also can act as an inverse agonist at the H_2 receptors, although under the conditions of this experimental protocol this would not be the case. Zolantidine easily crosses the BBB. By giving the drug before the trauma in these experiments, it was hoped to achieve maximal blocking of the H_2 receptors on the BBB and thereby obtain an optimal effect from use of the drug. The anticipated required doses were determined based on doses used in other zolantidine experiments. To the best of our knowledge no one has used zolantidine for BBB permeability studies previously, all of the studies used for reference were on neuronal H_2 mechanisms. It was also considered in the calculations that zolantidine was supposed to be 7-22 times as potent as traditional H_2 antagonists like cimetidine or ranitidine. The initial dose selected was 2.5 mg/kg. When this dose seemed to have no visible effect on the Evan's blue extravasation it was decided to go to higher doses. Because the amount of zolantidine available was limited, the dosage was increased to 10 and 20 mg/kg. This also limited the number of animals that could be used for the different dosage groups. The drug was reported to remain stable for up to 48 hours after preparing the solutions (with refrigeration between experimental sessions) (personal communication) but after that, any remaining drug in solution was not used. For these reasons, the number of animals in each dosage group was low. As it turned out the values for Evan's blue extravasation for the controls and for the mice in the 2.5 mg dosage group was very close, as were the results for the 10 mg and the 20 mg dosage groups. The

results were pooled into the two groups for purposes of data analysis. Comparison of the data revealed that treatment with zolantidine at 10 or 20 mg/kg reduced the Evan's blue extravasation, representing the increased permeability of the BBB, by 65%. The results indicated an impressive blocking effect for the zolantidine, although a stronger effect had been considered possible. It is not known whether this represents an optimal dose for this purpose. It would be necessary to test at higher doses as well. In addition, it should be tested for effectiveness at various time intervals after the trauma since other studies have shown the existence of a time window for intervening in the process of edema formation. The zolantidine is reported to have a good tolerability by animals in a wide range of doses up to at least 100 mg/kg. The assumptions made in determining the initial dosage may have been incorrect, although they were all the information available. It is possible the dosage that is required to block H_2 neuronal mechanisms in the CNS may require a lower dosage than blocking most of the H_2 receptors in the BBB. The BBB represents a large surface area with H_2 receptors. In addition, comparing the potency with cimetidine may have caused some miscalculation if the effects of cimetidine were the result of actions at more than just the H_2 receptors. Zolantidine was well tolerated by the mice and it did not alter the anesthetic requirements for isoflurane. One mouse that received a 10-mg/kg dose and was not able to complete the remainder of the experiment was returned to her home cage. Observation for several days afterward did not notice any overt changes in the health or behavior of the mouse.

Summary. This study has shown that a mild-moderate head trauma such as that used in these experiments does cause mast cell degranulation in the underlying dura. It has also shown that the degranulation is associated with increased cortical histamine in the

underlying cortex that is associated with increased permeability of the blood-brain barrier, a known pre-requisite for post-trauma edema formation. The experiments have also shown that blockade of the histamine H_2 receptors with zolantidine, at 10 and 20 mg/kg, can block up to 65% of the increase in permeability of the blood-brain barrier. This suggests that this drug should be further investigated for it's therapeutic potential in preventing post-trauma brain edema. The results also emphasize that a less severe head trauma can cause significant changes in permeability of the blood-brain barrier. This may cause a degree of post-trauma edema that contributes to the symptoms of MTBI as well and some of the resultant permanent damage. Further studies should consider these possibilities. Post-MTBI medical care could include the prescription of a drug like zolantidine for the first day or two after the trauma. A third potential application is for edema prevention in stroke patients. Stroke is the third leading cause of death in the United States, ranking only behind ischemic heart disease and all forms of cancer), and the leading cause of permanent disability (Devasenapathy and Hachinski 1999). A drug like zolantidine may improve recovery and reduce permanent neurological impairments.

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