Corticotropin-Releasing Hormone and Brain Mast Cells Regulate Blood-Brain-Barrier Permeability Induced by Acute Stress

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ABSTRACT

Stress activates the hypothalamic-pituitary-adrenal axis through release of corticotropin-releasing hormone (CRH), leading to production of glucocorticoids that down-regulate immune responses. Acute stress, however, also has proinflammatory effects that seem to be mediated through the activation of mast cells. Stress and mast cells have been implicated in the pathophysiology of various inflammatory conditions, including some in the central nervous system, such as multiple sclerosis in which disruption of the blood-brain barrier (BBB) precedes clinical symptoms. We previously showed that acute restraint stress increases rat BBB permeability to intravenous 99Tc gluceptate and that administration of the “mast cell stabilizer” disodium cromoglycate (cromolyn) inhibits this effect. In this study, we show that the CRH-receptor antagonist Antalarmin blocks stress-induced 99Tc extravasation, whereas site-specific injection of CRH in the paraventricular nucleus (PVN) of the hypothalamus mimics acute stress. This latter effect is blocked by pretreatment of the PVN with cromolyn; moreover, restraint stress cannot disrupt the BBB in the diencephalon and cerebellum of W/Wv mast cell-deficient mice. These results demonstrate that CRH and mast cells are involved in regulating BBB permeability and, possibly, brain inflammatory disorders exacerbated by acute stress.

The blood-brain barrier (BBB) is made up of brain microvessel endothelial cells (Johansson, 1990), astroglia, pericytes, perivascular macrophages, and basal lamina. Brain microvessel endothelial cells are characterized by tight intracellular junctions restricting passage of most molecules from the circulation to the brain. The protective function of the BBB can be altered during various disease states of the central nervous system, specifically during cerebral inflammation (De Vreis et al., 1997) such as that present in multiple sclerosis (MS) (Kermode et al., 1990). Brain leukocyte infiltration in MS (Smith and Weiner, 1997) follows a decrease in the integrity of the BBB (Kermode et al., 1990). BBB permeability may be affected by acute stress that seems to exacerbate symptoms in relapsing-remitting MS (Mei-Tal et al., 1970; Goodin et al., 1999).

Stress activates the hypothalamic-pituitary-adrenal (HPA) axis through the release of corticotropin-releasing hormone (CRH) leading to secretion of catecholamines and glucocorticoids; these, in turn, down-regulate the immune response (Chrousos, 1995). CRH is synthesized predominantly in the paraventricular nucleus (PVN) and mediates its effects through at least three types of receptors (CRHR): CRHR-1, CRHR-2α, and CRHR-2β. These are also present elsewhere in the brain indicating that CRH or structurally related compounds such as urocortin, which has stronger affinity for the CRH-R2 (Vaughan et al., 1995), might have paracrine actions. Stress, however, also worsens a number of neuroinflammatory disorders (Rosch, 1979), and CRH also has proinflammatory effects (Karalis et al., 1991), apparently mediated through mast cell activation (Theoharides et al., 1998). We recently reported that acute restraint stress increases BBB permeability in rats, an action dependent on mast cell activation because it was blocked by the “mast cell stabilizer” disodium cromoglycate (cromolyn) (Esposito et al., 2001).

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ABBREVIATIONS: BBB, blood-brain barrier; MS, multiple sclerosis; HPA, hypothalamic-pituitary-adrenal; CRH, corticotropin-releasing hormone; PVN, paraventricular nucleus; TNF-α, tumor necrosis factor-α; EAE, encephalomyelitis.
dependent (Theoharides et al., 1995). Moreover, CRH (Theoharides et al., 1998) induced mast cell degranulation and Evans blue extravasation in rodent skin, a phenomenon duplicated by acute restraint stress and also blocked by Antalarmin (Singh et al., 1999).

Mast cells are ubiquitous in the body and are critical for allergic reactions, but they also secrete numerous cytokines (Galli, 1993; Metcalfe et al., 1997). Increasing evidence indicates that mast cells may also be involved in neuroimmune interactions (Church et al., 1989; Rozniecki et al., 1999), including neuroinflammatory processes (Theoharides, 1996).

In the brain, mast cells are predominantly located perivascularly, especially in the thalamus and hypothalamus (Irbrahim, 1974; Pang et al., 1996). As many mast cell mediators are vasoactive, mast cells may regulate the BBB (Theoharides, 1990), a proposal supported by findings that a chemical trigger of mast cells, compound 48/80, increased BBB permeability in the mast cell rich habenula of pigeons (Zhuang et al., 1998). Mast cells are critical for many physiologic and pathologic conditions. For example, mast cells are involved in allergic reactions, but they also secrete numerous cytokines.

Materials and Methods

Restraint Stress and Indicator Extravasation. Male Sprague-Dawley, 300-g rats (Charles River, Wilmington, MA) were kept on a 14:10-h dark/light cycle and were provided food and water ad libium. Animals were first anesthetized with one i.p. injection (0.3 ml) of a mixture of ketamine and xylazine (1.0 and 0.02 ml, respectively, of 100 mg/ml each). They were then cannulated via the jugular vein. In certain cases, guide cannulas (Plastic One, Roanoke, VA) were inserted into the paraventricular region of the hypothalamus as described later. Animals were handled daily to check on the guide cannula and i.v. catheter to familiarize them with the investigators.

The morning of the experiment (9 AM–12 PM), animals were injected with 500 μCi of 99Tc gluceptate, which was prepared as follows. Gluceptate (DRAXIMAGE, Inc., Kirkland, Quebec, Canada), a d-glycerol-d-gluco-heptonate complex was obtained from Synchor Pharmacy (Woburn, MA). The gluceptate was then mixed with 99Tc (DuPont, Wilmington, DE) the morning of the experiment. Binding to gluceptate prevents 99Tc from escaping the circulation and constitutes a good maker of vascular permeability and extravasation in brain parenchyma (Jacobson et al., 1989). Control animals were left in their cages on the bench top for 30 min, but not in the presence of animals that were being stressed. Rats to be stressed were placed in a Plexiglas immobilizer (Harvard Apparatus, Cambridge, MA) immediately following 99Tc injection for 30 min in the laboratory. Separate groups of animals to be stressed were injected i.v. (0.2 ml) via the cannula with either 1.2, 4, or 10 mg/kg of the nonpeptide CRH receptor antagonist Antalarmin dissolved in Emulphor (Sigma-Aldrich, St. Louis, MO) 60 min before their placement in the stress chamber. Control animals received 0.2 ml of vehicle (Emulphor) through the same indwelling jugular catheter.

To assess BBB permeability, the animals were anesthetized with a single i.v. injection of 0.2 ml of ketamine and 0.05 ml of xylazine (100 mg/ml each) immediately after stress or at the indicated times. The heart was then perfused with a 60-ml syringe inserted into the left ventricle. The circulation was flushed with 60 ml of 0.9% NaCl followed by 120 ml of 10% formalin to remove any 99Tc trapped in the circulation that may contribute to a high background and to fix the tissue for light microscopy. Following perfusion, rats were decapitated, the whole brain was removed, and samples of the brainstem, cerebellum, cortex, and BBB permeability were administered. CRH centrally by an ipsilateral site injection in the PVN of the hypothalamus. We also provide further support that the stress-induced increase in BBB permeability requires mast cells since it is absent in mast cell-deficient mice.

Statistics. Due to the small half-life of 99Tc (about 6 h), it was impossible to assure delivery of exactly the same dose of 99Tc each day the experiment was performed. Therefore, it was necessary to express the difference of counts in stressed animals as a percentage of the controls from each day [experimental-control/mean control] × 100. We analyzed the data generated in two ways: 1) to establish
whether any change from baseline was statistically significant from zero, values were compared using a one-sample t test; and 2) to determine whether the change that occurred within treatment groups (e.g., with and without Antalarmin) was significant, values were compared using the nonparametric Mann-Whitney U test. For all tests of significance, \( \alpha \) was set at 0.05.

**Results**

**Effect of Restraint Stress on Serum Corticosterone.** Serum corticosterone levels were increased due to restraint stress in all experiments. Corticosterone levels in rats increased from 131.1 \( \pm \) 36.8 to 222.0 \( \pm \) 55.9 ng/ml with 30 min of restraint stress, as previously published (Esposito et al., 2001). Control C57BL mice had 55.0 \( \pm \) 24.9 ng/ml that increased to 386.4 \( \pm \) 57.2 ng/ml after 30 min restraint stress. The W/W\textsuperscript{v} mice had a similar response to stress with levels increasing from 40.5 \( \pm \) 35.9 to 317.3 \( \pm \) 47.0 ng/ml within 30 min (Huang et al., 2002). Therefore, any observed differences in BBB permeability in these mast cell-deficient mice were not due to differences in HPA axis activation.

**Effect of Antalarmin on \(^{99}\text{Tc}\) Extravasation.** BBB permeability was assessed by quantitating extravasation of \(^{99}\text{Tc}\) in brain parenchyma of the following four different brain areas to investigate any regional differences: diencephalon, cerebellum, cerebral cortex, and brainstem. Acute stress by restraint for 30 min increased BBB permeability in all brain regions, with the maximal increase of 210 \( \pm \) 130% noted in the cerebellum and the diencephalon that were statistically different from control (Fig. 1); the increase in the cortex, however, was not statistically significant. Pretreatment with Antalarmin (given i.v. at dosages of 1.2, 4, or 10 mg/kg body weight for 60 min before stress) reduced this effect in a dose-dependent manner in all brain regions studied (Fig. 1). Maximal reduction of \(^{99}\text{Tc}\) extravasation was observed in the diencephalon with the values dropping from 170 \( \pm \) 120% during stress to 7 \( \pm \) 25% after pretreatment with 10 mg of Antalarmin.

**Effect of CRH Injected into the PVN on \(^{99}\text{Tc}\) Extravasation.** We investigated whether intracranial administration of CRH could mimic the increased BBB permeability induced by acute stress. CRH first administered intravenicularly did not increase BBB permeability (results not shown). We then injected CRH into the PVN through a guide cannula implanted using stereotaxic co-ordinates. Histology of the injection site showed little pathology; the presence of a well organized compartment surrounding the track of the cannula suggests the tissue had recovered from the initial trauma caused by the implantation surgery (Fig. 2A). Figure 2B shows the location of the guide cannula tip (in the area of the PVN) in relation to the third ventricle. Site directed injection of CRH in the PVN increased BBB permeability about 50% in the diencephalon, cerebellum and cortex but not in the brainstem (Fig. 3).

**Effect of Cromolyn Injected into PVN on CRH-Induced \(^{99}\text{Tc}\) Extravasation.** To further investigate the involvement of mast cells, animals were injected with cromolyn into the PVN 30 min before CRH injection. Pretreatment

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**Fig. 1.** Effect of Antalarmin (1.2, 4, 10 mg/kg body weight) given i.v. 60 min before acute restraint stress (30 min) induced \(^{99}\text{Tc}\) extravasation in different brain regions (n = 5 rats/group). Values are compared using one sample t test. Asterisk (*) indicates \( p < 0.05 \). Double asterisk (**) indicates significance when treatment group is compared with the stress group using a Mann-Whitney U test. Di, diencephalon; Cbellum, cerebellum; Cort, cortex; Bstem, brainstem; Ant, Antalarmin.

**Fig. 2.** Histology of rat diencephalons using hematoxylin and eosin (H&E) stain. Diencephalic sections (10 \( \mu \)m) were stained and imaged under 400\( \times \). A, the guide cannula tract; B, arrow indicates the area just behind the tip of the cannula (in the intended injection site; PVN), with lighter staining suggesting mild neuronal loss; the arrow indicates the third ventricle.
with cromolyn reduced 99Tc extravasation significantly only in the diencephalon (Fig. 3).

**Effect of Acute Stress on 99Tc Extravasation in the Brain of W/W**

mast cell-deficient mice. W/W mast cell-deficient mice were shown to increase their serum corticosterone levels in response to 30 min of restraint stress equally to their wild-type controls (see above). Nevertheless, there was no 99Tc extravasation due to acute stress in any brain area, except for the brainstem, compared with their wild-type controls (Fig. 4). These results indicate that mast cells are critical for acute stress to increase BBB permeability.

![Graph showing % Change from Control for Di, Cbellum, Cortex, and Bstem regions with CRH and Cro+CRH conditions](image)

**Fig. 3.** Effect of pretreatment (5 min) with cromolyn (1 μl of 1 mM) injected in the PVN on 99Tc extravasation in various brain regions (n = 5) in response to similarly injected CRH (1 μl of 1 mM). Asterisk (*) indicates p < 0.05; double asterisk (**) indicates significance when treatment group is compared with the CRH group using the Mann-Whitney U test. Di, diencephalon; Cbellum, cerebellum; Cort, cortex; Bstem, brainstem; Cro, cromolyn.

![Graph showing % Change from Control for Di, Cbellum, Cortex, and Bstem regions with C57 and W/W** conditions](image)

**Fig. 4.** Effect of acute stress (30 min) on 99Tc extravasation in brain regions of C57BL mice (A) (n = 10) or W/W mast cell-deficient mice (B) (n = 10). Asterisk (*) indicates p < 0.05. Double asterisk (**) indicates significance when W/W is compared with C57BL using Mann-Whitney U test. Di, diencephalon; Cbellum, cerebellum; Cort, cortex; Bstem, brainstem.

### Discussion

To our knowledge, this is the first time that CRH is shown to be involved in BBB permeability induced by acute stress, which is supported by the fact that stress-induced increase in BBB is blocked by the CRHR antagonist Antalarmin and that it is mimicked by the administration of CRH in the PVN of the hypothalamus. Although CRH is typically thought to be expressed in the hypothalamus, it is also detected in extrahypothalamic sites; these include the central and medial nuclei of the amygdala, the olfactory bulb, the cortex, and the deep cerebellar nuclei of the cerebellum (Dieterich et al., 1997). CRH activates the HPA, but may have other central effects because CRHR are expressed in other brain parts. CRHR-1 expression is highest in the cerebral cortex, striatum, amygdala, and cerebellum (Chalmers et al., 1996), whereas CRHR-2 is present mostly in subcortical structures such as the lateral septal nucleus, several nuclei of the hypothalamus, and the choroid plexus (Chalmers et al., 1996). It was previously suggested that CRH may be involved in dura mast cell activation in response to restraint stress (Theoharides et al., 1995) and skin mast cell activation and vascular permeability (Theoharides et al., 1998) since these effects were blocked by the CRHR-antagonist Antalarmin (Theoharides et al., 1998; Rozniecki et al., 1999). Antalarmin has higher selectivity for the CRHR-1 (Webster et al., 1996) and has been shown to block stress-induced behavioral effects (Deak et al., 1999).

Even though site injection of CRH increased BBB permeability, when CRH was administered i.c.v., it was ineffective. It is possible that CRH is cleared from the ventricular system before reaching the brain parenchyma; for instance, a saturable efflux allows CRH to be transported of the brain into the blood (Martins et al., 1997). The fact that CRH administered into the PVN increased 99Tc extravasation in other brain regions (cerebellum, brainstem, and cortex) suggests different possibilities: 1) CRH could diffuse outside the diencephalon and have local (paracrine) effects; 2) CRH could affect neurons in the diencephalon that project into other regions of the brain, possibly leading to neuronal release of vasoactive compounds such as substance P or TNF-α; and 3) mediators from activated diencephalic mast cells could have effects elsewhere. This last possibility is less likely due to the fact that pretreatment with cromolyn injected directly into the PVN-blocked 99Tc extravasation only in the diencephalon. Cromolyn either may not be able to diffuse to all areas where CRH reaches or may not block mast cell activation completely, especially since it does not block all types of mast cells (Fox et al., 1988). Alternatively, cromolyn may not only be blocking histamine release in the diencephalon, mostly responsible for BBB permeability, but also released cytokines that diffuse to other brain areas and increase BBB permeability; cromolyn may be able to inhibit the release of some cytokines, as it has been shown to inhibit TNF-α production from rat mast cells (Bissonnette et al., 1995) and passively sensitized human lung (Matsuo et al., 2000). Nevertheless, that 99Tc extravasation in the diencephalon was inhibited by pretreatment with site-injected cromolyn confirms that this process requires mast cells, at least in the diencephalon. This premise is also supported by the complete absence of any 99Tc extravasation in this region in W/W mast cell-deficient mice. The diencephalon is the brain area...
with the highest number of mast cells (Ibrahim, 1974; Pang et al., 1996), whereas the cerebellum contains a smaller number (Powell et al., 1999). Mast cells are localized around the cerebral microvasculature (Robinson-White and Beaver, 1982) and have also been identified close to CRH-positive neurons in the rat median eminence (Theoharides et al., 1995). CRH may be acting directly on mast cells, as it was also recently shown that mast cells express CRHR-1 and -2 (Sugimoto et al., 2002).

The involvement of mast cells in BBB permeability is also supported by reports that the mast cell secretagogue compound 48/80 stimulated brain mast cells in rats (Dimitriadou et al., 1990) and in pigeons (Zhuang et al., 1996). Moreover, local application of 48/80 to pia-induced BBB permeability to fluorescein-labeled dextran (Mayhan, 2000), whereas histamine increased BBB permeability as shown with 99mTc-te-289odium pertechnetate or 131I-serum albumin (Boertje et al., 1989), as well as by transendothelial electrical resistance in brain microvessels (Butt and Jones, 1992). Both histamine and serotonin may be involved in rodents, as pretreatment with the mixed histamine-serotonin receptor antagonist cypriohedrine inhibited BBB permeability induced by forced swimming (Sharma et al., 1991). The vasodilatory and proinflammatory TNF-α (Galli, 1993) could also be involved since this cytokine is released along with histamine from rat hypothalamic mast cells and has been shown to regulate BBB permeability (Kim et al., 1992). In fact, TNF-α was reported to be increased in the cerebrospinal fluid of MS patients (Hartung et al., 1995), and interference with TNF function prevents encephalomyelitis (EAE) (Klinkert et al., 1997).

The present results further our understanding of the regulation of BBB permeability and its involvement in neuroinflammatory diseases (De Vreis et al., 1997). For instance, the diencephalon, where we documented maximal BBB permeability, is involved in MS and could be a sufficient starting point through which mast cell-derived molecules could affect global BBB integrity (Rozniecki et al., 1995). Breakdown of BBB integrity has been documented to precede any clinical symptoms or pathological findings in MS (Kermode et al., 1990), and symptoms in relapsing-remitting MS often appear to worsen by psychological stress (Mei-Tal et al., 1970; Goodin et al., 1999). Therefore, it is relevant that acute restraint stress significantly shortened the onset of experimental allergic encephalomyelitis (EAE) (Klinkert et al., 1997).

Our results indicate that the effect of acute stress on BBB permeability is mediated through CRH and brain mast cells, but not that mast cells regulate the HPA axis. In fact, recent findings indicate that certain behavioral responses to stress still occur in CRH knockout mice (Jacobson et al., 2000; Muglia et al., 2001). Nevertheless, the mast cell secretagogue compound 48/80 was shown to increase serum corticosterone levels through activation of hypothalamic mast cells (Gadek-Michalska et al., 1991). Moreover, immunological stimulation of hypophalamic mast cells also led to HPA axis activation and serum corticosterone elevation (Matsumoto et al., 2001), prompting the speculation that mast cells may have a much more versatile role than previously suspected (Gurish and Austen, 2001). Mast cells could either induce CRH release or some hypothalamic mast cell mediator could independently activate the HPA axis. For instance, histamine and interleukin-6 can stimulate CRH release (Kjaer et al., 1998), and interleukin-6 has been shown to be a CRH-independent activator of the HPA axis (Bethin et al., 2000). Taken together, these results indicate that there are bidirectional actions of CRH on mast cells and such interactions (Rozniecki et al., 1999) could contribute in diseases exacerbated by stress (Theoharides, 2002).

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References


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