

# 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  $^{99}\text{Tc}$  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  $^{99}\text{Tc}$  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  $W/W^y$  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-2 $\alpha$ , and CRHR-2 $\beta$ . These are also present elsewhere in the brain indicating that CRH or structurally related compounds such as urocortin, which has stronger affinity for the CRHR-2 (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). Acute restraint stress was shown to induce intracranial rat mast cell activation and elevate cerebrospinal fluid levels of rat mast cell protease (RMCP-I), actions that were CRH-

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**ABBREVIATIONS:** BBB, blood-brain barrier; MS, multiple sclerosis; HPA, hypothalamic-pituitary-adrenal; CRH, corticotropin-releasing hormone; PVN, paraventricular nucleus; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; 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 (Ibrahim, 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., 1996).

In this study, we show that BBB permeability induced by acute restraint stress involves CRH because it is blocked by pretreatment with Antalarmin and is mimicked by site-directed CRH injection in 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.

## 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 libitum. 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 and to familiarize them with the investigators.

The morning of the experiment (9 AM–12 PM), animals were injected with 500  $\mu$ Ci of  $^{99}\text{Tc}$  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  $^{99}\text{Tc}$  (DuPont, Wilmington, DE) the morning of the experiment. Binding to gluceptate prevents  $^{99}\text{Tc}$  from escaping the circulation and constitutes a good marker of vascular permeability and extravasation in brain parenchyma (Jacobson et al., 1989). Control animals were left in their cage 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  $^{99}\text{Tc}$  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  $^{99}\text{Tc}$  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 diencephalon were collected as follows. After the cerebellum was removed, the spinal cord and brainstem (containing pons and medulla) were separated; the frontal pole of the cerebral hemispheres included gray matter and the underlying white matter. The diencephalon was isolated after removal of the mid-brain, the cerebral hemispheres, basal ganglia, and septum; it consisted of the internal capsule, thalamus, hypothalamus, subthalamus, and epithalamus. Blood was collected at the time of the intracardiac injection for corticosterone measurements. The tissue samples were then weighed, and the amount of radioactivity was expressed as counts per 100 mg of tissue. Control animals for each experiment were injected with the same amount of  $^{99}\text{Tc}$ , but were left in their cages on the same bench away from the presence of the animals being stressed.

Based upon preliminary data, this study was designed to detect at least a 50% difference in  $^{99}\text{Tc}$  uptake in the diencephalon, with a desired power of at least 90%. Five animals per group were needed given the variability observed. Each figure represents data generated from independent experiments.

**Use of W/W<sup>v</sup> Mice in  $^{99}\text{Tc}$  Extravasation Experiments.** Male C57BL mice, as well as W/W<sup>v</sup> mast cell-deficient mice (WBB 6F1/J-W/W<sup>v</sup>) and their wild-type controls (8 weeks old), were obtained from Jackson Labs (Bar Harbor, ME) and were kept four per cage. Mice were handled daily for 5 days to acclimate them to the investigators. On the morning of the experiment, mice were injected with 0.2 ml of 75  $\mu$ Ci  $^{99}\text{Tc}$  via the tail vein. Following injection, mice were either placed in their home cages or immobilized for 30 min. After this time, mice were perfused and decapitated, and their brains were removed as described for rats above. It was estimated that 10 mice per group were necessary for 50% increase in BBB permeability of the diencephalon due to stress.

**Site-Specific Injections.** Following anesthesia, rats were placed in the stereotaxic apparatus and prepared as described above. Guide cannulas (Plastic One) were inserted into the paraventricular region of the diencephalon as follows: 1.80 mm lateral and 1.90 mm posterior from Bregma, at a 10° angle and 8.9 mm deep (Paxinos and Watson, 1986). The animals were allowed to recover in the animal facility for 14 days before use. Animals were handled daily to check the guide cannula and i.v. catheter and to familiarize them with the investigators. To determine whether exogenous CRH could mimic the effect of stress on BBB permeability, we administered CRH centrally by an ipsilateral site injection in the PVN of the hypothalamus. The injection was unable to target any specific subnuclei because of the size of the cannula. We chose to inject the PVN because most of the endogenous CRH is localized in this area and mast cells are plentiful in the median eminence, close to CRH positive neurons (Pang et al., 1996). Animals with implanted guide cannulas received 1  $\mu$ l of 1 mM CRH (5  $\mu$ g) or 1  $\mu$ l 0.9% NaCl directly in the PVN. Animals remained in their cages for 30 min and were not restrained.

To determine whether the effect of CRH injected into the diencephalon was through mast cells, the same site in the diencephalon was pretreated with 1  $\mu$ l of 1 mM cromolyn for 30 min before CRH administration. Control animals were pretreated with 1  $\mu$ l 0.9% NaCl.

**Corticosterone Measurements.** Corticosterone was measured in the serum of both rats and mice using an ImmuChem double-antibody corticosterone 125-RIA kit (ICN Biomedicals, Costa Mesa, CA).

**Statistics.** Due to the short half-life of  $^{99}\text{Tc}$  (about 6 h), it was impossible to assure delivery of exactly the same dose of  $^{99}\text{Tc}$  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]  $\times$  100. We analyzed the data generated in two ways: 1) to establish

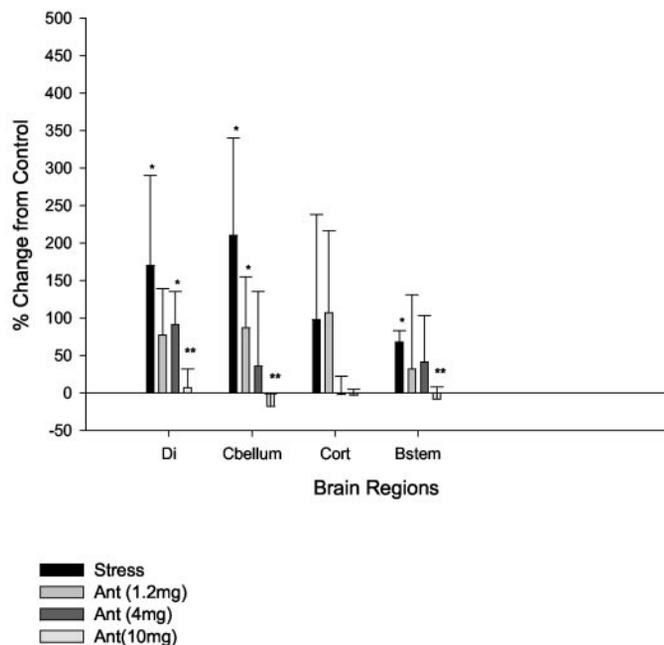
whether any change from baseline was statistically significant from zero, values were compared using a one-sample *t* test; and 2) to determine whether the change that occurred within treatment groups (e.g., with and without Antalarmin) was significant, values were compared using the nonparametric Mann-Whitney *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<sup>v</sup> 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 <sup>99</sup>Tc Extravasation.** BBB permeability was assessed by quantitating extravasation of <sup>99</sup>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).

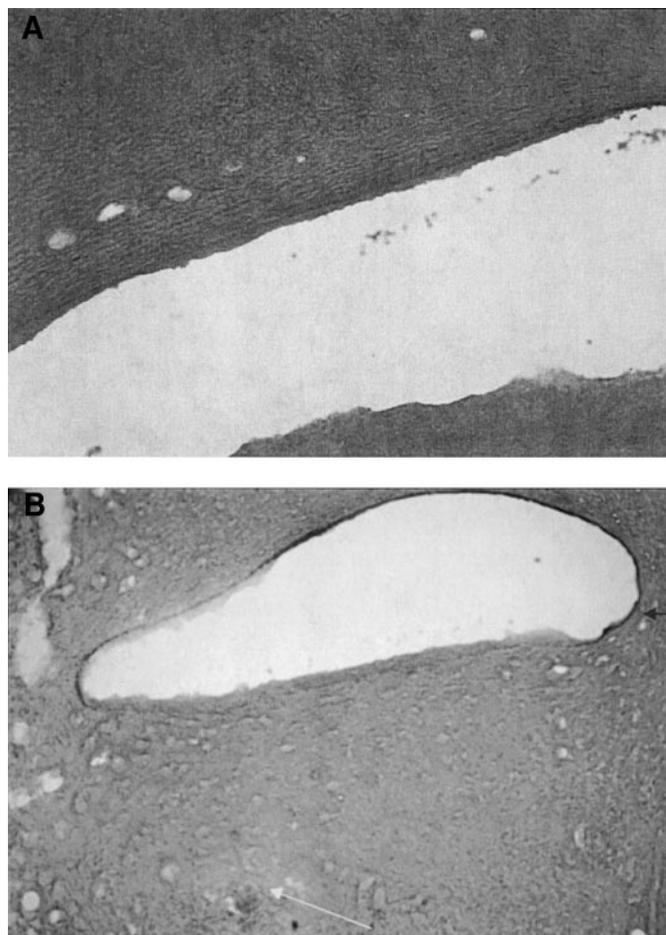


**Fig. 1.** Effect of Antalarmin (1.2, 4, 10 mg/kg body weight) given i.v. 60 min before acute restraint stress (30 min) induced <sup>99</sup>Tc extravasation in different brain regions ( $n = 5$  rats/group). Values are compared using one sample *t* test. Asterisk (\*) indicates  $p < 0.05$ . Double 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.

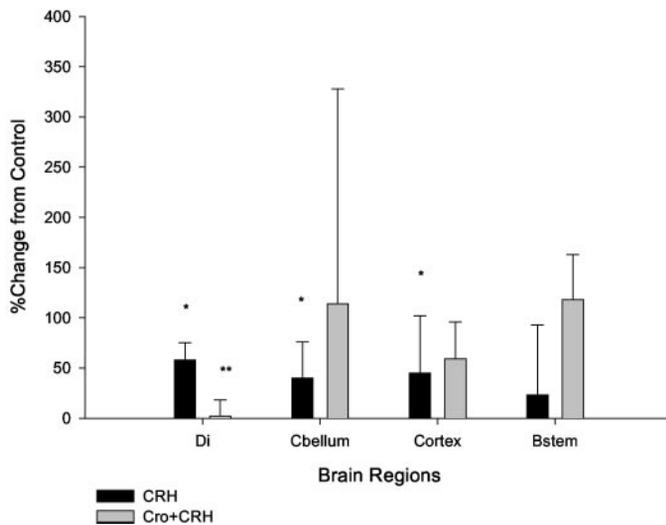
Maximal reduction of <sup>99</sup>Tc extravasation was observed in the diencephalon with the values dropping from  $170 \pm 120\%$  during stress to  $7 \pm 25\%$  after pretreatment with 10 mg of Antalarmin.

**Effect of CRH Injected into the PVN on <sup>99</sup>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 <sup>99</sup>Tc Extravasation.** To further investigate the involvement of mast cells, animals were injected with cromolyn into the PVN 30 min before CRH injection. Pretreatment



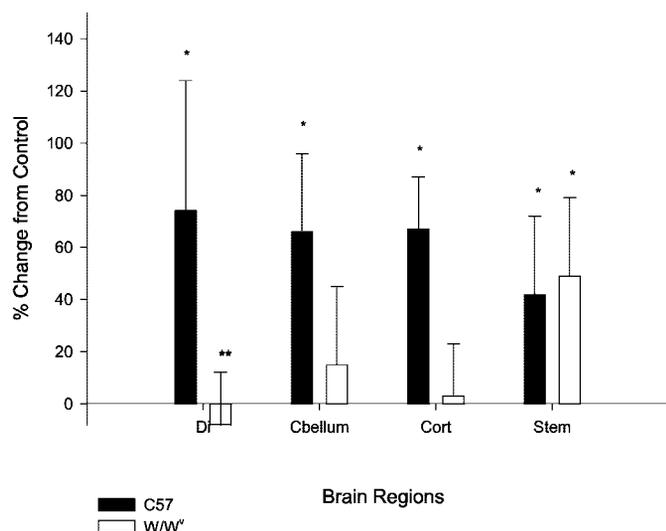
**Fig. 2.** Histology of rat diencephalons using hematoxylin and eosin (H&E) stain. Diencephalic sections ( $10 \mu\text{m}$ ) were stained and imaged under  $400\times$ . A, the guide cannula tract; B, arrow indicates the area just behind the tip of the cannula (in the intended injection site; PVN),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



**Fig. 3.** Effect of pretreatment (5 min) with cromolyn (1  $\mu$ l of 1 mM) injected in the PVN on  $^{99m}\text{Tc}$  extravasation in various brain regions ( $n = 5$ ) in response to similarly injected CRH (1  $\mu$ l 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.

with cromolyn reduced  $^{99m}\text{Tc}$  extravasation significantly only in the diencephalon (Fig. 3).

**Effect of Acute Stress on  $^{99m}\text{Tc}$  Extravasation in the Brain of W/W<sup>v</sup> Mast Cell-Deficient Mice.** W/W<sup>v</sup> 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  $^{99m}\text{Tc}$  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.



**Fig. 4.** Effect of acute stress (30 min) on  $^{99m}\text{Tc}$  extravasation in brain regions of C57BL mice (A) ( $n = 10$ ) or W/W<sup>v</sup> mast cell-deficient mice (B) ( $n = 10$ ). Asterisk (\*) indicates  $p < 0.05$ . Double asterisk (\*\*) indicates significance when W/W<sup>v</sup> 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  $^{99m}\text{Tc}$  extravasation in other brain regions (cerebellum, brainstem, and cortex) suggests several 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- $\alpha$ ; 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  $^{99m}\text{Tc}$  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- $\alpha$  production from rat mast cells (Bissonnette et al., 1995) and passively sensitized human lung (Matsuo et al., 2000). Nevertheless, that  $^{99m}\text{Tc}$  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  $^{99m}\text{Tc}$  extravasation in this region in W/W<sup>v</sup> 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 Beaven, 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  $^{99m}\text{Tc}$ -sodium pertechnetate or  $^{131}\text{I}$ -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 cyproheptadine inhibited BBB permeability induced by forced swimming (Sharma et al., 1991). The vasodilatory and proinflammatory TNF- $\alpha$  (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- $\alpha$  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 EAE in rats (Chandler et al., 2002). EAE has been associated with increased and activated hypothalamic mast cells (Dimitriadou et al., 2000), while the severity of EAE was reduced and the onset delayed in W/W<sup>v</sup> mast cell mice (Secor et al., 2000).

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, immunologic stimulation of hypothalamic 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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