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Brain Research Protocols 8 (2001) 143–149

BRAIN RESEARCH PROTOCOLS

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Protocol

Non-invasive assessment of blood–brain barrier (BBB) permeability using a gamma camera to detect ^{99}technetium-gluceptate extravasation in rat brain

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Accepted 13 July 2001

Abstract

The blood–brain barrier (BBB) is a complex structure of endothelial cells, astroglia, pericytes, and perivascular macrophages enclosed by basal lamina. The BBB regulates the entry of blood-borne molecules and cells into the brain, but it is disrupted in various inflammatory conditions of the central nervous system (CNS). We previously showed that 30 min of immobilization stress increased ^{99}technetium-gluceptate (^{99}Tc) extravasation, measured by a gamma counter, in brain regions containing mast cells, an effect blocked by the mast cell stabilizer disodium cromoglycate [Brain Res. 888 (2001) 117]. Here we report the use of a gamma camera to assess BBB permeability by assessing ^{99}Tc extravasation in the rat brain, during and following acute stress, without having to sacrifice the experimental animals. This method also allows for repeated experimentation on the same animal, since the half-life of ^{99}Tc is only 6 h, and permits testing of potential inhibitors of BBB permeability.

Theme: Cellular and Molecular Biology

Topic: Blood-brain barrier

Keywords: Stress; Imaging; Mast cell; Multiple sclerosis; AIDS

1. Type of research

- Non-invasive assessment of BBB permeability using gamma camera imaging of ^{99}Tc. The use of the gamma camera not only permits real time measurements of brain ^{99}Tc levels, but the short half-life of ^{99}Tc (6 h) permits the repeated use of the same animals. Previous studies investigating BBB permeability used either a fluorescence tracer [29], ^{131}I-albumin [4] or ^{131}I-sodium [23] extravasation: however, these studies require removal of brain tissue and can only investigate single time-points.
- An example of application of this protocol is semi quantitation of ^{99}Tc extravasation in whole brain during and after stress.

2. Time required for execution of the protocol

- Implantation of jugular vein catheters to deliver ^{99}Tc= 30 min/animal.
- Gamma Counter imaging acquisition=120 min (different lengths of time are possible).
- Downloading of imaging data to PC processing=2 h.

3. Materials

3.1. Preparation of animals

- Male Sprague–Dawley 300 g rats.
- Surgical equipment for implantation of jugular catheters.
- Silastic tubing (for jugular catheter) size=0.025 inches I.D.×0.047 inches O.D.

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1385-299X/01/$ – see front matter
PII: S1385-299X(01)00104-0

3.2. To assess ^{99}Tc extravasation

- Gluceptate (DRAXIMAGE Inc, Kirkland, Quebec, Canada).
- ^{99}Tc (obtained from Dept. of Health Physics, New England Medical Center, Boston, MA, USA).
- Sigma 400 Radioisotope Nuclear Gamma Camera (Ohio Nuclear, Solon, OH, USA).
- Macintosh computer and Macintosh Gamma Camera software:Gamma Camera Acquisition and Control Image Display and Analysis Version 1.0b4 (Streichman Medical Equipment, Medfield, MA, USA).

4. Detailed procedure

4.1. Application — preparation of animals and ^{99}Tc

Male Sprague–Dawley 300 g rats (Charles River, NY, USA) 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 ml and 0.02 ml, respectively, of 100 mg/ml each). They were then cannulated via the jugular vein. The animals were allowed to recover in the animal facility for 5 days prior to use. Animals were handled daily to check on the intravenous catheter and familiarize them with the investigators. The morning of the experiment (9.00 a.m.–12.00 p.m.), animals were injected with 500 μCi of ^{99}Tc which was prepared as follows: (1) Gluceptate (DRAXIMAGE, Kirkland, Quebec, Canada), a D-glycero-D-gluco-heptonate complex was obtained from Synchor Pharmacy (Woburn, MA, USA); (2) the gluceptate was then mixed with ^{99}Tc (DuPont, Billerica, MA, USA). The binding of gluceptate prevents ^{99}Tc from escaping the circulation and constitutes a good marker from extravasation in brain parenchyma [11].

The accumulation of ^{99}Tc in the whole brain was followed with the aid of a Sigma 400 Radioisotope Nuclear Gamma Camera (Ohio Nuclear). A maximum of four rats could be imaged simultaneously on the camera. Images and data were collected each minute for 90–120 min. The whole brain was selected as the 'region of interest' and the counts per mm^2 (intensity of radiation) were quantified by a Macintosh Gamma Camera software program: Gamma Camera Acquisition and Control Image Display and Analysis, version 1.0b4 (Streichman Medical Equipment). Raw counts versus time were plotted for each animal. The area under the curve (AUC) was calculated by the computer using the trapezoid rule and the data was expressed as percent change from control AUC. Values were compared using a one sample Student's *t*-test. The study was designed to detect at least 50% difference in ^{99}Tc uptake in the whole brain with a desired power of at least 90%. Four animals per group were used in all dynamic studies. These studies were approved by the Tufts University Animal Research Committee.

Immediately following ^{99}Tc injection, stress animals were restrained for a specified amount of time using a plexiglas immobilizer (Harvard Apparatus, Cambridge, MA, USA); they were then anesthetized and placed on the camera for imaging. Control animals remained in their cages for a similar time and were then anesthetized and imaged. In separate experiments, experimental animals were stressed by restraining on the camera using surgical tape (Fig. 1); this variation allowed for measurements to be taken during the stress period. Control animals were also anesthetized and imaged.

4.1.1. Duration of immobilization time required to increase BBB permeability

Rats were injected with ^{99}Tc and then either placed back in their cage (control) or in the immobilizer for 15 min, 30 min or 60 min. Following the stress period, all animals were immediately anesthetized and placed on the camera. As a maximum of four animals can be imaged simultaneously on the camera, two control animals were always imaged along with two experimental animals. Images and data were collected each minute for 90–120 min. The amount of brain ^{99}Tc (counts/mm^2) was then quantified.

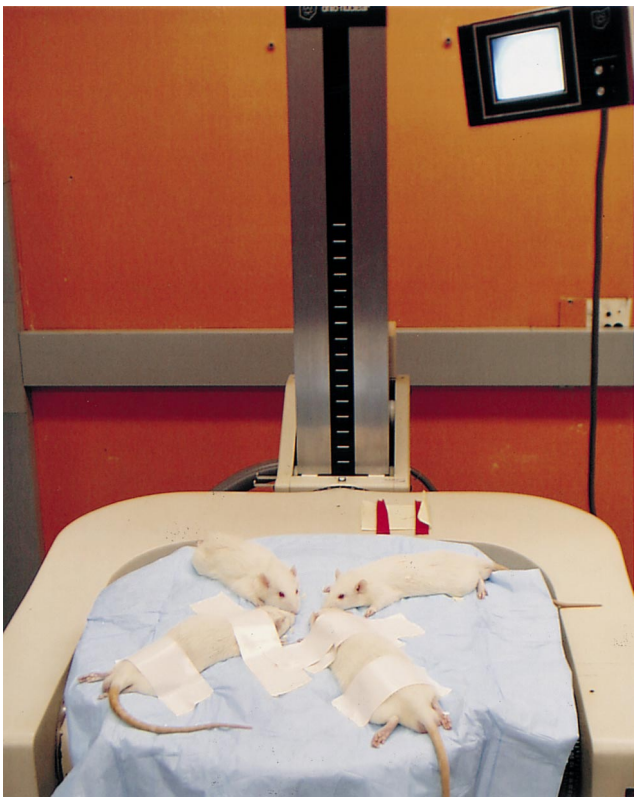

Fig. 1. Photograph of the gamma camera set-up showing position and method of restraint of the experimental animal.

4.1.2. How soon after immobilization can changes in ^{99}Tc extravasation be detected?

In order to determine just how soon after the start of restraint one could identify BBB permeability changes, unanesthetized rats were imaged while they were being stressed. These animals were restrained on the camera plate for 30 min using surgical tape (Fig. 1). Within 5 min of imaging, all rats were injected with ^{99}Tc via the indwelling catheter. Control animals were anesthetized and also placed on the camera for imaging. At the end of 30 min, all animals were anesthetized and removed from the camera.

4.2. Immobilization stress and ^{99}Tc extravasation in distinct brain regions

All animals were injected with ^{99}Tc as detailed above. Control animals were left in their cage on the bench top for 30–120 min (depending on the experiment), but not in the presence of animals that were being stressed. Rats to be stressed were placed in a plexiglas immobilizer immediately following ^{99}Tc injection in the laboratory for the designated period of time. The animals were then handled as described previously [8]. In brief, the animals were anesthetized, perfused to remove any ^{99}Tc in the circulation and then decapitated. Brain regions were collected and counted using a gamma well counter.

4.3. Statistics

Due to the short half-life of ^{99}Tc (about 6 h), it was impossible to assure delivery of exactly the same dose of ^{99}Tc each day the experiment was performed. It was thus necessary to express the difference of counts in stressed animals as a percent of the controls from each day (experimental−control/mean control)×100. For gamma camera studies, the AUC for each animal was calculated with the trapezoid rule using Excel. Data is expressed as percent change from control AUC and compared using a one-sample *t*-test. A one-sample *t*-test is commonly used to compare one experimental group to baseline which is taken as zero. Significance is denoted by $P<0.05$. In addition, differences between the various stress groups (i.e. 15, 30, 60 min stress) were compared to each other using non-parametric, Mann–Whitney *U*-test.

5. Results

5.1.1. ^{99}Tc extravasation investigated by gamma camera in whole brain following stress

Real time evaluation of ^{99}Tc was obtained using a gamma camera. The results show increased uptake immediately after stress. Fig. 2 is a representative graph of brain images of four animals (two controls and two stressed) taken each minute. Following 30 min stress, the whole brain contained more ^{99}Tc than control animals for up to approximately 100 min during which time radioactivity progressively decreased (Fig. 2). This experiment was repeated three times with qualitatively similar data (data not shown). Fig. 2 (insert) shows a set of brain images obtained by the gamma camera 33 min from ^{99}Tc injection.

Animals were then stressed for different periods of time and then placed on the camera for 90 min in order to investigate the length of stress required to induce maximal ^{99}Tc extravasation in the brain. Mean AUC were compared and shows that 15, 30 or 60 min of immobilization increased ^{99}Tc extravasation to a roughly equivalent extent (Fig. 3). After 15 min of immobilization, ^{99}Tc increased by 70.1% compared to control ($n=4$, $P=0.002$), while after 30 min, the increase was 116.6%. After 60 min of stress, ^{99}Tc increased 120.6% compared control (Fig. 3). However, there was no significant difference between the 30 min, the 15 or 60 min values.

5.1.2. ^{99}Tc extravasation investigated by gamma camera in whole brain during stress

Results from the previous section indicated that there was no significant difference in the ^{99}Tc accumulation between 15, 30 and 60 min of immobilization. We therefore restrained animals using tape on the camera in order to measure brain ^{99}Tc from the start of the stress period and up to 45 min. Fig. 4 is a representative graph of the ^{99}Tc levels in the brain of a control animal and an animal being restrained on the gamma camera (four animals per group were used). It is obvious that by 8 min of immobilization (5 min from ^{99}Tc injection), the brain of the restrained animal contained more ^{99}Tc that control. This increase was maximal by 10 min from immobilization. It appeared that the ^{99}Tc increase in the stressed animal persisted for up to 20 min following immobilization, irrespective of the decline of both curves over time. When AUCs are generated and compared, the increase in ^{99}Tc extravasation in stress versus control is comparable to that reported in the previous section for animals stressed for 30 min (Fig. 3).

5.2. ^{99}Tc extravasation measured by gamma well counter

Whole brain ^{99}Tc measurements with the gamma camera includes counts in the skin, meninges and cerebral vasculature. We, therefore, investigated (1) whether ^{99}Tc counts in the brain after the removal of skin, dura and vasculature still reflected the gamma camera findings, and (2) in which parts of the brain ^{99}Tc extravasated. Animals stressed similarly were sacrificed at designated times

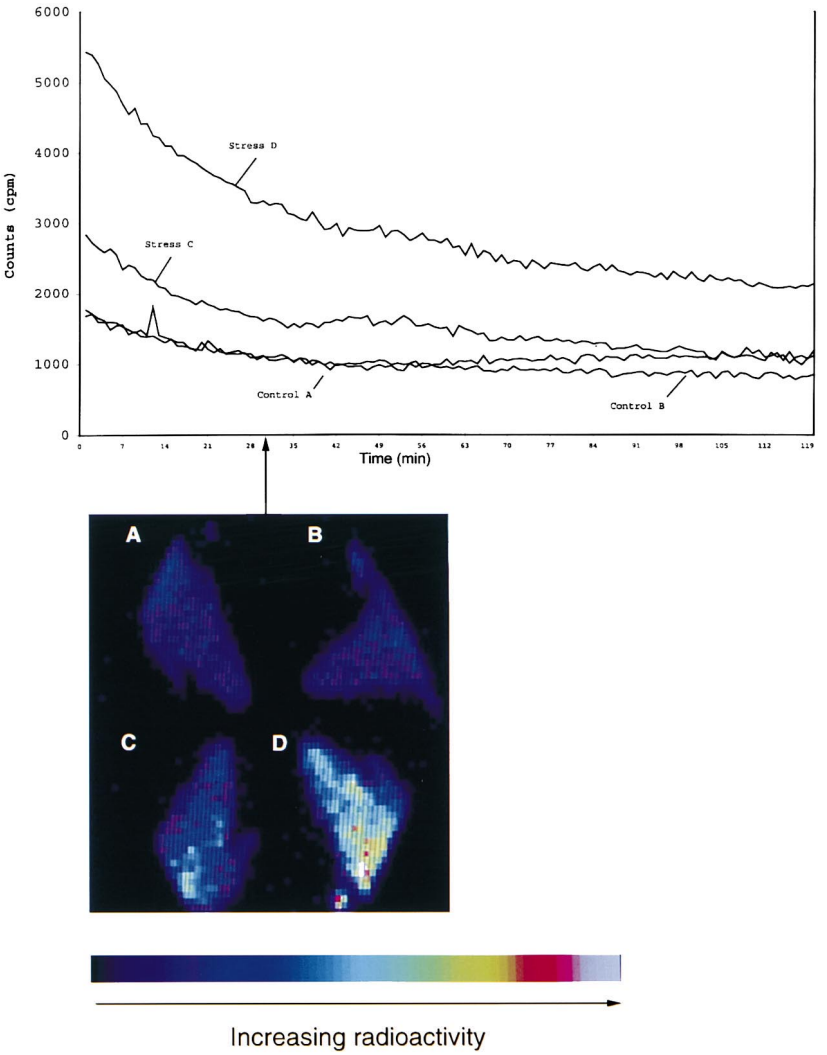

Fig. 2. Real time dynamic study of ^{99}Tc levels in the brain using a gamma camera ($n=4$) following 30 min stress by immobilization. Vertical line indicates the time-point from which the brain images shown on the insert were obtained. (A, B) represent two control animals, while (C, D) are two animals stressed for 30 min.

following the 30 min stress period and distinct brain regions were dissected out and counted in a gamma well counter. Extravasation of ^{99}Tc in brain parenchyma was studied at 0, 45 and 90 min after animals were subjected to 30 min immobilization stress (Fig. 5). Control animals were exposed to ^{99}Tc for the same period. All animals were then deeply anesthetized and perfused with 10% neutral buffered formalin. Four different brain areas were isolated and then evaluated to investigate any regional differences: (a) brainstem, (b) cerebellum, (c) cerebral cortex, and (d) diencephalon.

Maximal increase in ^{99}Tc extravasation, as percent change (from control) immediately after 30 min of stress, was evident in the diencephalon, followed by the cerebellum and the brainstem. A similar effect was not seen in the cerebral cortex. While all brain regions showed increases over time, ^{99}Tc extravasation significantly increased only in the diencephalon for up to 90 min following acute stress.

6. Discussion

The gamma camera allows non-invasive real time measurements of brain ^{99}Tc levels to investigate BBB permeability and is relevant to human disease because it is the marker of choice in such studies [17,24]. This approach allows minute to minute assessment of ^{99}Tc levels in the brain and permits estimation of: (a) the time of maximal activity/the peak level and (b) how long the ^{99}Tc stays in the brain. In contrast to the gamma counter study, the short half-life of ^{99}Tc (6 h) makes the repeated use of the same animals possible, thus allowing the same animal to act as its own control and reducing the number of animals

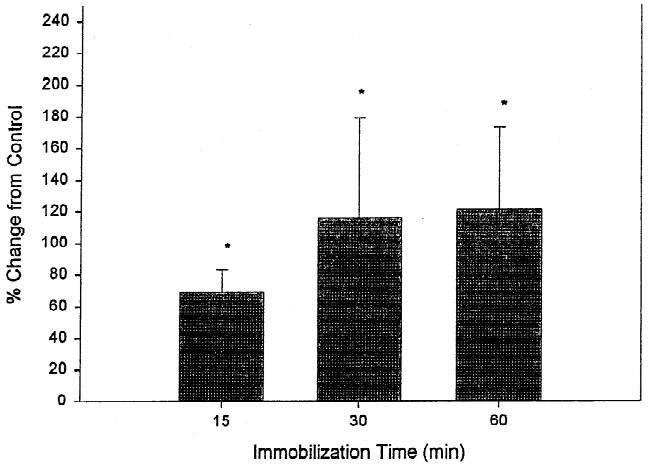

Fig. 3. Quantitation of ^{99}Tc by gamma camera in the brain of rats stressed for the times shown. Results ($n=4$ for each group) are presented as percent change of AUC from control for 120 min following the designated duration of stress. Significance is denoted by an asterisk ($P<0.05$).

necessary. ^{99}Tc binds with high affinity to gluceptate and was used because the technetium glucoceptonate complexes [19] remain in the vasculature long enough to permit studies of induced extravasation [11]. It is used clinically to assess renal perfusion and does not accumulate in brain unless there is an impaired BBB. In contrast, technetium-99m-pertechnetate is used to assess first pass circulation in the brain, but over 20% becomes free ^{99m}Tc that leaks out of the circulation by 2 h, making it unsuitable for investigating BBB integrity [14]. However, we noted considerable inter-animal variability, as shown in Fig. 2, in response to stress; similar variability was also noted when using a gamma well counter. We believe this variability is due to individual responses to stress and is not attributable to the technique described here. It is important to note that variation among control animals was very small. It is only in the stress group that there is large variation (apparent in Fig. 2).

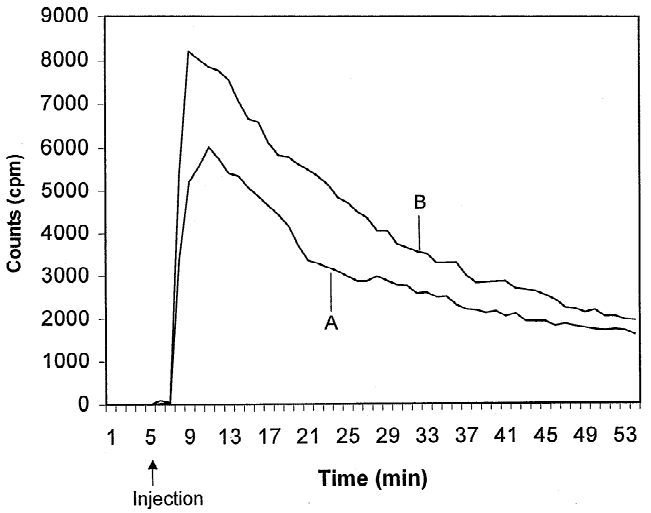

Fig. 4. Real time dynamic study of ^{99}Tc levels in the brain of (A) a control rat placed on the gamma camera after it was anesthetized, and (B) a rat taped on the gamma camera for the duration shown.

The changes observed after stress using the gamma camera are somewhat greater than that observed in isolated brain regions (90% increase) using the gamma counter [8]. Immobilization stress increased BBB permeability to ^{99}Tc by as much as 120% as shown by the gamma camera studies. This discrepancy could be explained by the presence of some ^{99}Tc in the skin over the skull, meninges and vasculature when using the gamma camera; as in the gamma camera studies, the brain has been removed from the skull after intracardiac washing of ^{99}Tc from the circulation. Another possible explanation could be the contribution of blood pressure and blood flow since there might be greater perfusion and possible extravasation if these were higher. However, it has been reported that 5–15 min of immobilization actually did not change systemic blood pressure and caused regional cerebral blood flow to decline by about 13% [20]. Nevertheless, changes in blood volume due to other experimental conditions could affect the results. There may also be some contribution of the anesthetic used to permit imaging. However, the use of ketamine/xylazine did not affect ^{99}Tc uptake in the brain of control animals; moreover, our data show that whether rats were first stressed before they were anesthetized and imaged, or restrained by surgical tape on the gamma camera pad without anesthetic, the extent of ^{99}Tc extravasation was equivalent (as evidenced by similar AUCs). Even though anesthetics could alter blood flow and serum corticosterone levels, the published data are confusing. For instance, one paper reported that halothane decreased serum corticosterone levels in rats [5] while another one reported the opposite [12].

Improved understanding of the regulation of the BBB [3] will help identify means by which the endogenous mechanisms can be tapped for deliberate control of BBB permeability. Experimental manipulation of the BBB may have therapeutic value as short-term increases in permeability could permit drug delivery to the brain, while inhibition of BBB permeability could reduce the damaging effects of brain inflammation [2]. An example of the latter would be multiple sclerosis (MS) where increase in the BBB permeability has been shown to precede any pathological or clinical evidence of disease activity [13,15,25], especially in MS exacerbations which appear to be precipitated by acute stress [9,16,28]. Similarly, BBB breakdown is considered crucial to the entry of T-cell or macrophages harboring HIV in AIDS patients with neurologic symptoms [6,21]. In both these conditions, brain mast cells may play a critical role as neither experimental allergic encephalomyelitis [22] nor virus-produced encephalitis [10,18] develop in mast cell deficient mice.

Vasoactive molecules released from mast cells such as

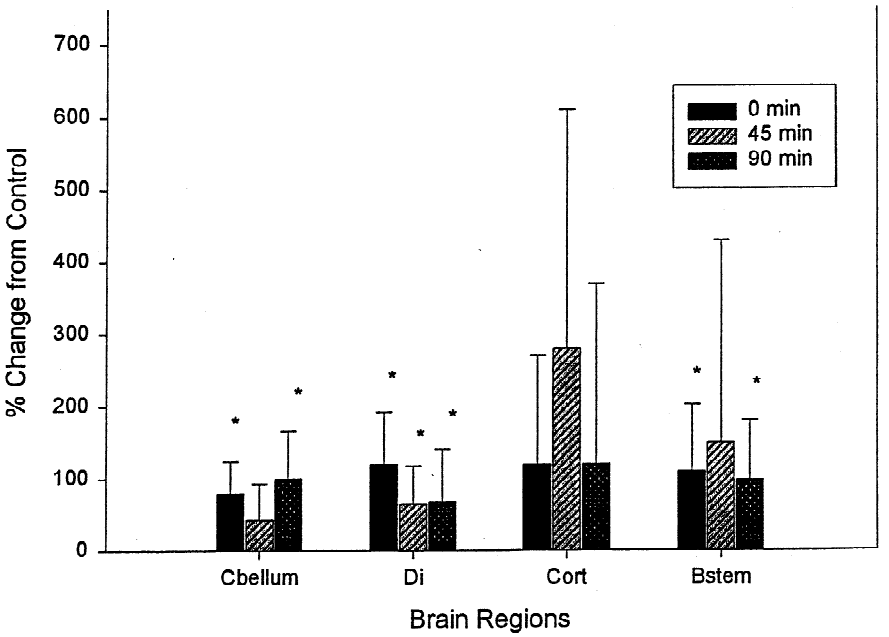

Fig. 5. Effects of acute immobilization stress on ^{99}Tc extravasation reported as percent change in various brain regions over time as shown: Cbellum=cerebellum, Di=diencephalon, Cort=cerebral cortex, Bstem=brainstem. In each case, comparisons were made to the control. Comparisons among the different lengths of times following stress were not significant. Statistical significance ($P<0.05$) is denoted by an asterisk ($n=5$ rats per group; representative graph of one of three experiments).

histamine, TNF-α, vasoactive intestinal peptide, nitric oxide, or interleukin-6 [26] could affect permeability of the BBB [1,30]. Administration of these compounds or their antagonists/blockers could provide possible therapeutic interventions. For instance, we showed that acute stress increases BBB permeability through corticotropin-releasing hormone (CRH) acting directly or through brain mast cell activation [7]. In fact, a CRH receptor antagonist prevented acute stress-induced intracranial mast cell activation [27]. Using this protocol, one can investigate select drugs for their effects on BBB permeability and may provide information on the mechanism and/or mediators involved.

Acknowledgements

This work was supported in part by NIH grant NS38326 to TCT. Thanks are due to Dominic Siewko for his help with making ^{99}Tc timely available. We also thank Sharon Titus and Yvonne Battad for their word processing skills.

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