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# DEFINITIVE CHARACTERIZATION OF RAT HYPOTHALAMIC MAST CELLS

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**Abstract**—Mast cells have previously been identified in mammalian brain by histochemistry and histamine fluorescence, particularly in the rat thalamus and hypothalamus. However, the nature of brain mast cells has continued to be questioned, especially because the electron microscopic appearance often shows secretory granule morphology distinct from that of typical connective tissue mast cells. Here we report that mast cells in the rat hypothalamus, identified based on metachromatic staining with Toluidine Blue, fluoresced after staining with berberine sulfate, indicating the presence of heparin. These cells were also positive immunohistochemically for histamine, as well as for rat mast cell protease I, an enzyme characteristically present in rat connective tissue mast cells. In addition, these same cells showed a very strong signal with *in situ* hybridization for immunoglobulin E binding protein messenger RNA. However, use of antibodies directed towards immunoglobulin E or its binding protein did not label any cells, which may mean either the binding protein is below the level of detection of the techniques used or that it is not expressed except in pathological conditions when the blood–brain barrier becomes permeable. At the ultrastructural level, perivascular mast cells contained numerous, intact, electron-dense granules which were labeled by gold-labeled anti-rat mast cell protease I.

These results clearly demonstrate the presence of perivascular mast cells in the rat hypothalamus, where they may participate in homeostatic processes. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

*Key words*: brain, hypothalamus, histamine, immunoglobulin E, mast cells, mast cell protease I.

Mast cells were first recognized by Friedrich von Recklinghausen in 1863 and were named by Paul Ehrlich in 1878 when their granules were stained metachromatically purple with Toluidine Blue. They have since been shown to store histamine, cytokines and proteolytic enzymes, such as rat mast cell protease (RMCP) I and II, which identify connective tissue mast cells (CTMCs) and mucosal mast cells (MMCs), respectively.[18] CTMCs are further differentiated from MMCs because the former contain heparin, while the latter store other sulfated proteoglycans.[18] Mast cells are known to leave the bone marrow as immature cells which then mature under microenvironmental conditions.[17] However, mast cells show plasticity since even bone marrow-derived cells and MMCs can differentiate into CTMCs when grown together with 3T3 cells.[17] Mast cells have also been found in the brain,[11,20,24] where they may regulate vascular permeability and inflammatory cell entry in the brain parenchyma.[52]

Brain mast cells (BMCs) were identified morphologically mostly by Toluidine Blue staining or histamine fluorescence with *o*-phthaldialdehyde in the leptomeninges,[14] thalamus[20] and hypothalamus,[11,14,24] as well as by histamine immunohistochemistry in the median eminence.[40] A low ratio of histidine decarboxylase to histamine content measured by a microradioenzymatic technique also suggested the presence of mast cells in rat hypothalamus.[43] Moreover, the mast cell secretagogue compound 48/80 (C48/80) reduced the histamine content in this region, suggesting that a portion of the histamine was likely stored in mast cells.[43]

With the *o*-phthaldialdehyde condensation method and Toluidine Blue staining, mast cells were localized almost exclusively in the rat thalamus[20] and it was concluded that they resemble CTMSs, but with less histamine content.[20] These latter studies estimated that about 50% of total brain histamine (excluding the dura, which is rich in mast cell histamine) and possibly almost 90% of thalamic histamine come from mast cells.[19] However, the same authors reported that the brain of W/W$^v$ mast cell-deficient mice had as much histamine as their +/+ controls.[23] In addition, it was shown elsewhere that some of the mouse brain histamine may be of non-neuronal,

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*Abbreviations*: BBB, blood–brain barrier; BMC, brain mast cell; BSA, bovine serum albumin; C48/80; CTMC, connective tissue mast cell; EDTA, ethylenediaminetetraacetate; FcεRI, immunoglobulin E binding protein; FITC, fluorescein isothiocyanate; IgE, immunoglobulin E; ISH, *in situ* hybridization; MMC, mucosal mast cell; PB, phosphate buffer; RBL, rat basophil leukemia; RMCP-I, rat mast cell protease I; SSC, saline sodium citrate; TBS, Tris-buffered saline.

non-mast cell origin, suggesting the existence of a distinct compartment.[38] Still other studies indicated that the same W/W$^v$ mice had only 45% of brain histamine compared to their controls.[59] Moreover, many BMCs stained with Sudan Black,[8,24] indicating the presence of lipid, which differentiated them from CTMCs or MMCs[24] and led to the suggestion that some BMCs be termed neurolipomastoid (Type II cells).[26] Finally, the ultrastructural appearance of activated BMCs is quite distinct from that of CTMCs in that it is primarily characterized by intragranular changes without typical compound exocytosis.[8,26] For these reasons, there continues to be considerable skepticism about the nature and significance of BMCs and their relationship to the neurolipomastoid cells.

## EXPERIMENTAL PROCEDURES

### *Tissue preparation*

Male Sprague–Dawley rats weighing about 200 g (Taconic, Germantown, NY, U.S.A.) were anesthetized with ether and perfused with intracardiac administration of 50 ml saline followed by 100–150 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). All efforts were made to minimize animal suffering and to reduce the number of animals used. The whole hypothalamus or the dura were removed and postfixed in 4% paraformaldehyde at 4°C for 6 h. For berberine staining only, the rats were perfused using 100–150 ml of a solution containing 40% formaldehyde, glacial acetic acid and absolute methanol (1:1:8 by volume), following which the hypothalamus was removed and postfixed in formaldehyde–acetic acid–methanol for 12 h. All tissues were then soaked in 20% sucrose in PB at 4°C overnight and frozen in tissue freezing medium (Cat. no. H-TFM, Triangle Biomedical Sciences, Durham, NC, U.S.A.). Frozen sections were cut at 7 $\mu$m with a cryostat (Jung CM 3000, Leica, Deerfield, IL, U.S.A.).

Rat homogeneic peritoneal mast cells were purified as described previously.[58] After purification, mast cells were centrifuged (170 $\times$ *g* for 5 min at 24°C) and the pellet was fixed in 4% paraformaldehyde for 30 min at room temperature, followed by three washes in PB for 10 min each. The cells were then frozen in freezing medium and cut at 7 $\mu$m with a cryostat as before. Rat basophil leukemia (RBL) cells were grown in stationary cultures as described previously.[56]

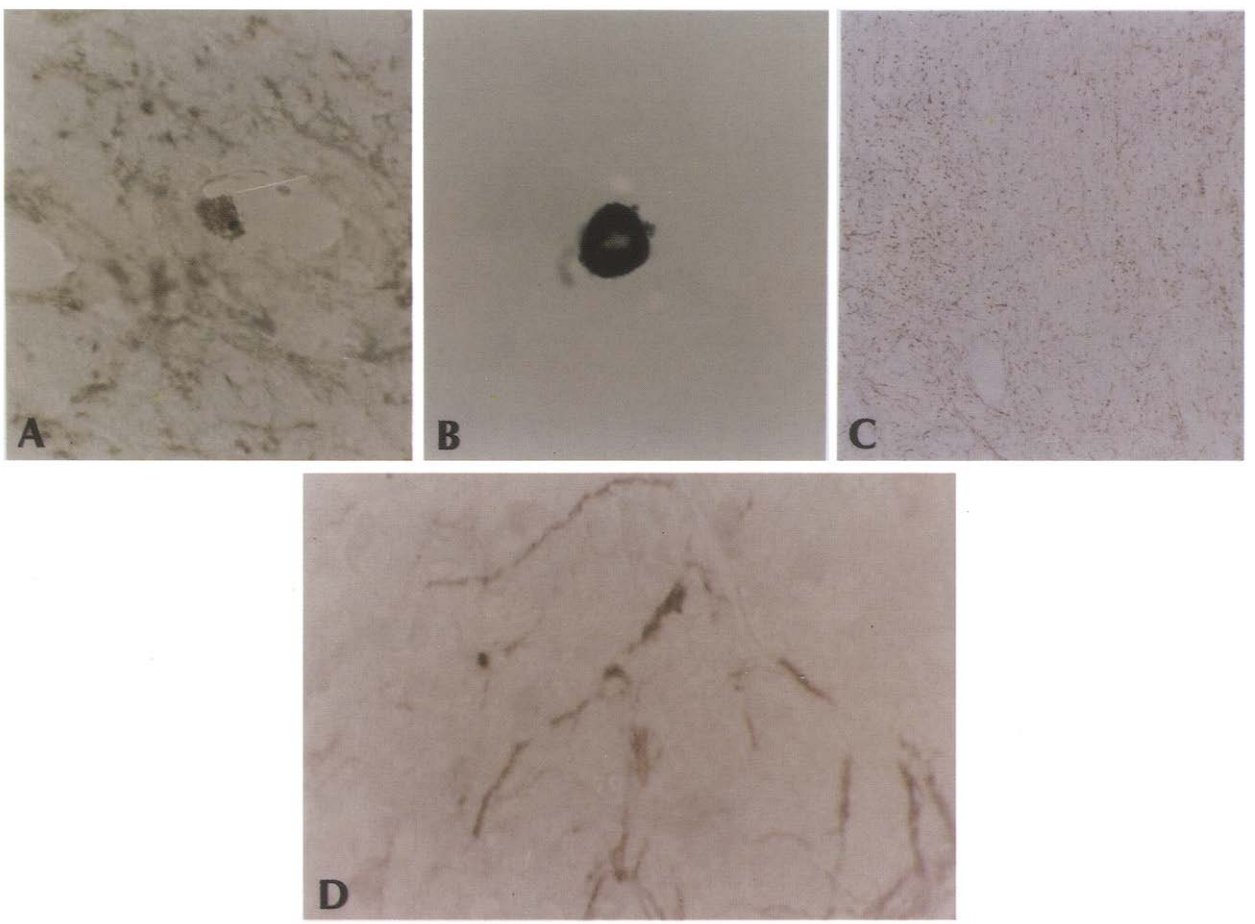


Fig. 1. Photomicrograph showing histamine immunoreactivity processed with avidin–biotin conjugated peroxidase complex in: (A) mast cell from the hypothalamus (note histamine localization in mast cell granules) and (B) homogeneic, purified peritoneal mast cell. Magnification for A and B: $\times$1000. (C) Negative control with antibody preabsorbed with histamine showing no immunoreactivity in the hypothalamus. (D) Positive control showing histamine-immunoreactive nerve processes in the hypothalamus. Magnification: $\times$200.

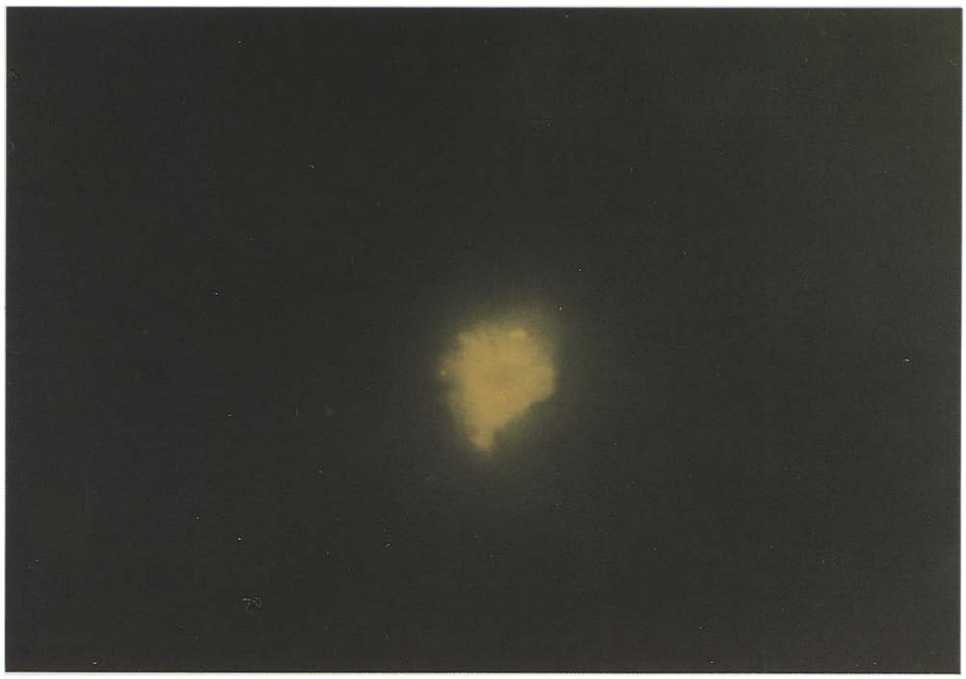

Fig. 2. Photomicrograph showing berberine sulfate staining of a heparin-containing mast cell in the hypothalamus. Magnification: × 1000.

In situ *hybridization and characterization*

*Probe preparation.* Custom-designed antisense/sense oligodeoxynucleotide probes were prepared using an Applied Biosystems automated DNA synthesizer at Tufts University School of Medicine. For the selective detection of immunoglobulin E binding protein (FcεRI) mRNA, a 48-base sequence (419–467) complementary to the region of cDNA[1] was selected (antisense probe): 5′ CAT GCG AGG CAT GAC TCC TCC AGG CAA GGG CAT ATC GTA GGG CAC TGT 3′. The complementary sense probe served as the control.

The 3′ tailing oligonucleotides were generated with digoxigenin-11-dUTP/dATP standard oligonucleotide tailing reaction using a Genius 6 kit (Boehringer Mannheim, Mannheim, Germany). Digoxigenin-labeled probes were quantified by a dot blot procedure with the Genius 1 kit (Boehringer Mannheim), both according to the manufacturer's recommendations.

*Northern analysis.* The specificity of the probe was documented by a single band on northern blot analysis using RNA extracted from RBL cells which are known to express (FcεRI,[2] as well as by the lack of staining of homogeneic rat cerebellum, which is known to be devoid of mast cells.[31]

Total RNA was purified from RBL cells with TRIzol reagent (Gibco BRL, Gaithersburg, MD, U.S.A.). The RNA (16 μg) was denatured for electrophoresis on 1% agarose gel containing 2.2 M formaldehyde and then transferred to nitrocellulose.[47] Sense and antisense FcεRI oligonucleotides were labeled with $^{32}P$ using terminal transferase for hybridization (Gibco BRL).

*Tissue pretreatment.* The slides were brought to room temperature, rinsed in PB and then immersed in 0.2 N HCl for 20 min followed by a wash in PB; the slides were then placed in 1 g/ml Proteinase K (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 30 min. After three washes in PB, the slides were put in 4% paraformaldehyde for 30 min, washed in PB and then immersed in 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min followed by a wash in PB and sequentially dehydrated in 50, 70, 95% ethanol and chloroform.

*Hybridization.* The slides were incubated with prehybridization buffer which contained 48.6% deionized formamide (Sigma), 150 mg/ml dextran sulfate (Sigma), 0.25 mg/ml yeast tRNA (Sigma), 20 μl/ml Denhardt's solution (Sigma), 10 ng/ml polyadenosine (Sigma), 20 units/ml polydeoxyadenosine (Sigma), 1 μg/ml Randomer (Dupont), 50 μl/ml salmon sperm DNA (Sigma) and 0.1 M dithiothreitol in PB for 2 h at 37°C. The addition of polyadenosine, polydeoxyadenosine and Randomer was used to reduce background, as shown previously.[33]

After removal of excess prehybridization solution, *in situ* hybridization (ISH) was initiated by application of labeled probes (2 pmol/slide) in 250 μl of hybridization buffer, which was identical to the prehybridization buffer, under a coverslip. The slides were incubated at 37°C overnight under low stringency conditions. After hybridization, the slides were washed twice each with 2 × saline sodium citrate (SSC) and 1 × SSC containing 43% deionized formamide for 15 min each at room temperature. Two stringent washes, each of 30 min, were carried out at 37°C in 0.25 × SSC containing 33% deionized formamide. This was followed by rinsing in 300 mM ammonium acetate.

*Immunological detection.* The slides were treated essentially according to the procedure provided by Boehringer Mannheim for the revelation of digoxigenin-labeled nucleic acids (Boehringer Mannheim) with small modifications as follows: (i) wash for 5 min in 0.1 M Tris–HCl, 150 mM NaCl, pH 7.5 (Buffer 1); (ii) incubation with 2% normal sheep serum for 1 h at room temperature; (iii) incubation with sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) at 1:250 dilution for 3 h at room temperature; (iv) two washes of 10 min each in Buffer 1; (v) 10 min equilibration in 0.1 M Tris–HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5 (Buffer 2); (vi) color development with freshly prepared substrate solution containing 0.175 mg 5-bromo-4-chloro-3-indolylphosphate and 0.37 mg nitroblue tetrazolium salt per ml of Buffer 2. This step was performed in the dark for 24 h at room temperature. When the colorimetric reaction was completed, the slides were rinsed in 10 mM Tris–HCl, 1 mM

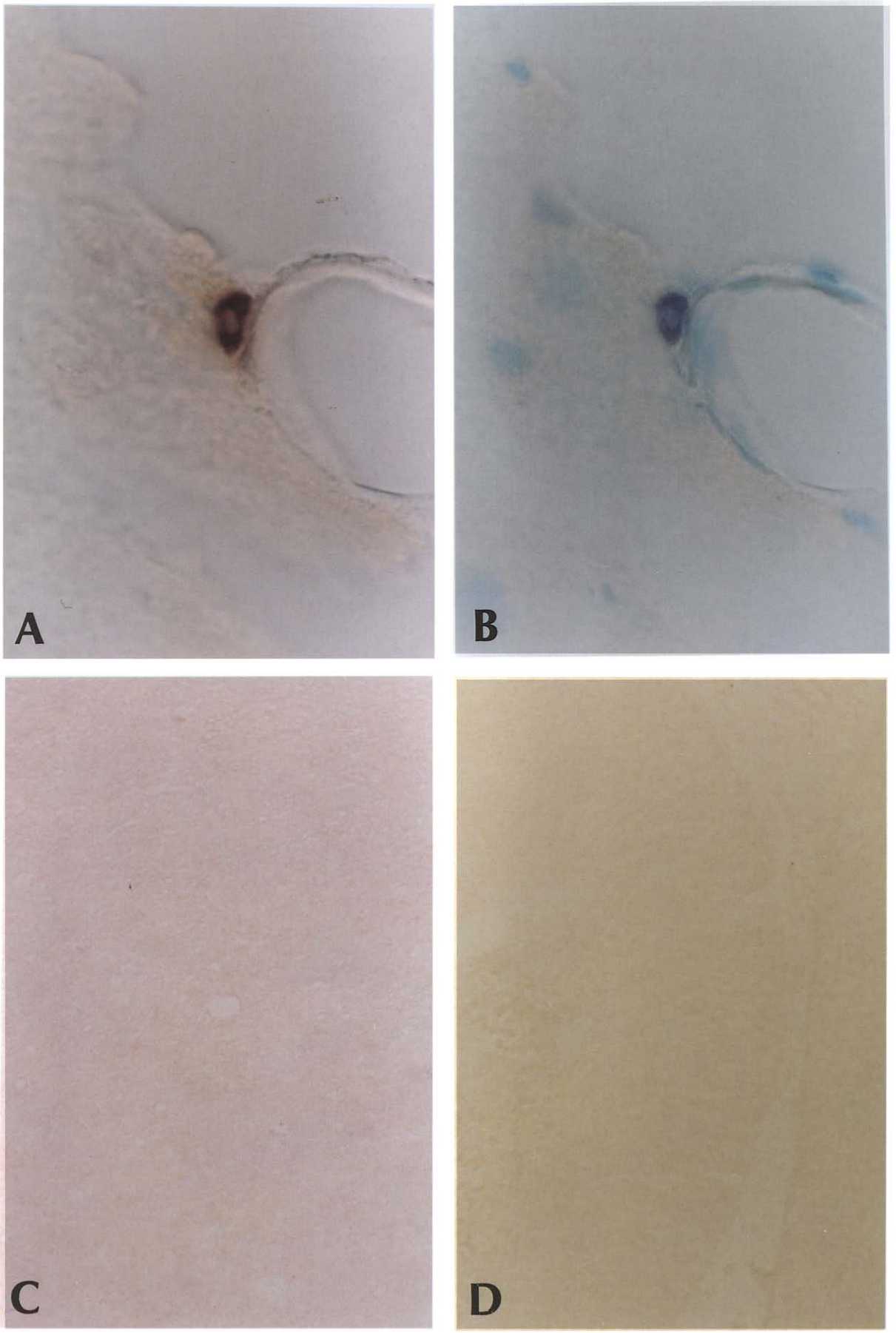


Fig. 3(A–D). (*caption opposite*)

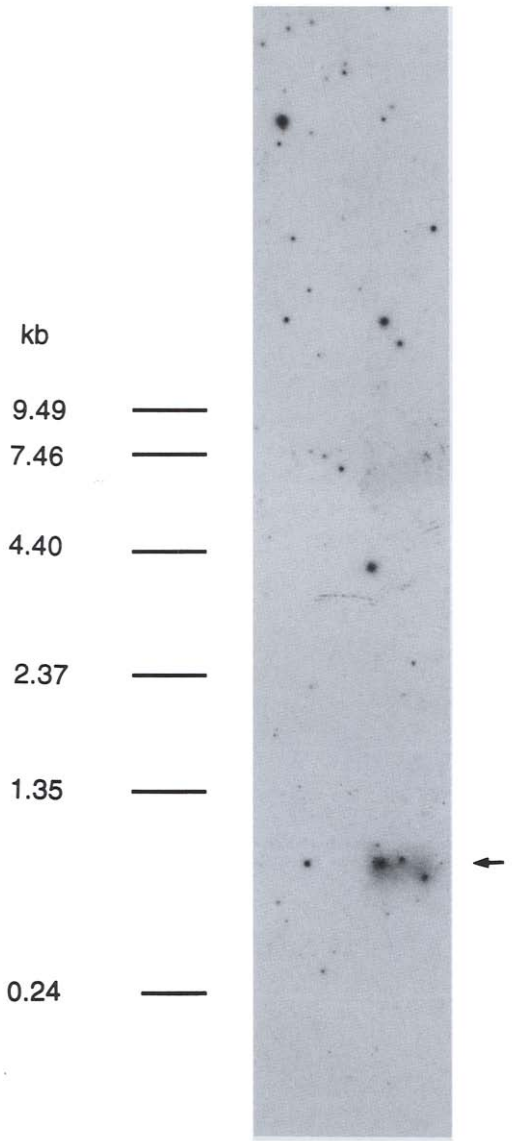


Fig. 3. Photomicrographs of rat hypothalamus probed by ISH for rat FcεRI mRNA using a digoxigenin-11-UTP-labeled (A) antisense DNA probe showing a mast cell adjacent to a blood vessel with the hybridization signal restricted to the cytoplasmic portion. Magnification: ×1000. (B) The same section counterstained with Toluidine Blue demonstrating it to be a mast cell. Magnification: ×200. (C) A hypothalamic section stained with sense DNA probe. Magnification: ×200. (D) Rat cerebellum stained with the same antisense probe used in A showing no signal. (E) Northern blot performed as described in Experimental Procedures: lane A, RNA size markers visible under ultraviolet light; lane B, total RBL RNA. Magnification: ×200.

EDTA, pH 8.1 (Buffer 3), then dipped in distilled water and mounted in aqueous mounting medium (Biomeda, Foster City, CA, U.S.A., Cat. no. M01).

The slides were observed and photographed with an inverted Diaphot light microscope (Nikon, Don Santo Carp., Natick, MA, U.S.A.).

*Immunohistochemistry*

*Rat mast cell protease I.* The slides (−50°C) were allowed to air dry at room temperature for 5 min. All subsequent steps were also performed at room temperature. After the slides were rinsed briefly in PB, they were treated with 5% normal donkey serum for 30 min followed by incubation with sheep antibody to rat mast cell protease I (RMCP-I; Moredun Animal Health, Edinburgh, U.K.) at 1:500 dilution for 1 h. For secondary antibody, the slides were incubated with biotin-conjugated donkey anti-sheep immunoglobulin G (Chemicon International, Temecula, CA, U.S.A.) at 1:100 dilution for 30 min. After three washes in PB, the slides were incubated with streptavidin–fluorescein isothiocyanate (FITC; Pierce, Rockford, IL, U.S.A.) at 1:200 dilution for 30 min, they were washed

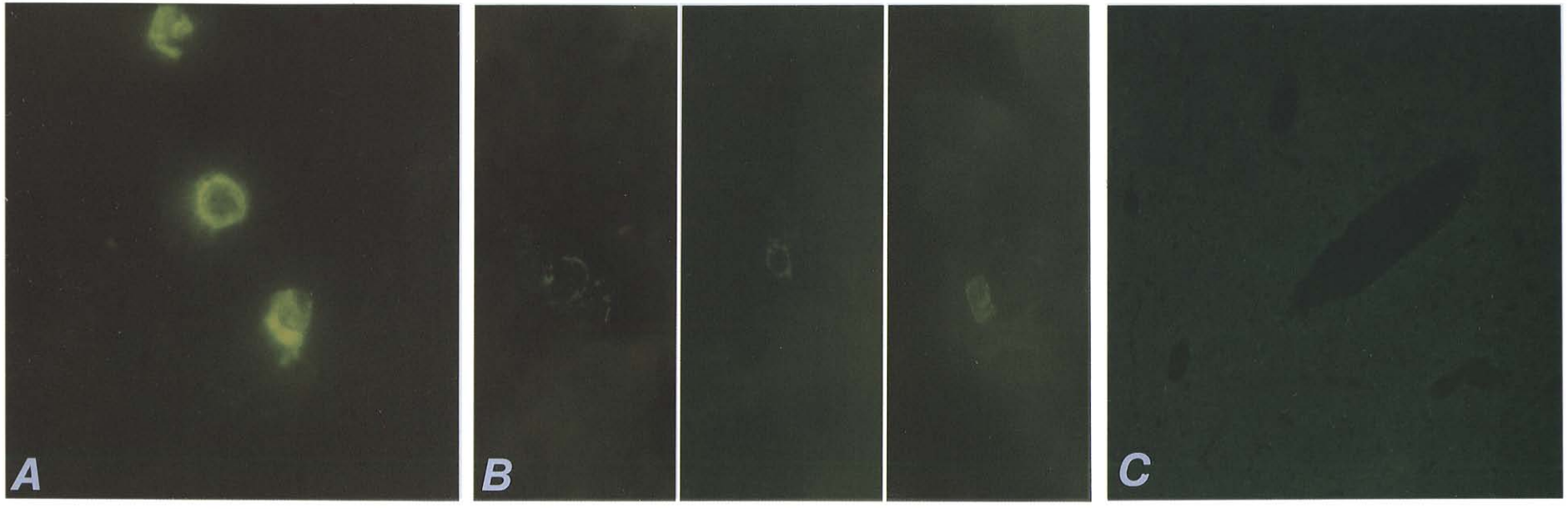


Fig. 4. Photomicrographs of tissue specimens stained with fluorescein-labeled monoclonal mouse antibody to rat IgE. (A) Purified peritoneal mast cells. Magnification: × 1000. (B) Homogeneic dura mast cells. Magnification: × 1000 (dura mast cells are smaller than peritoneal and look even smaller due to the plane of sectioning. (C) Homogeneic hypothalamus showing no positive cells. Magnification: × 100.

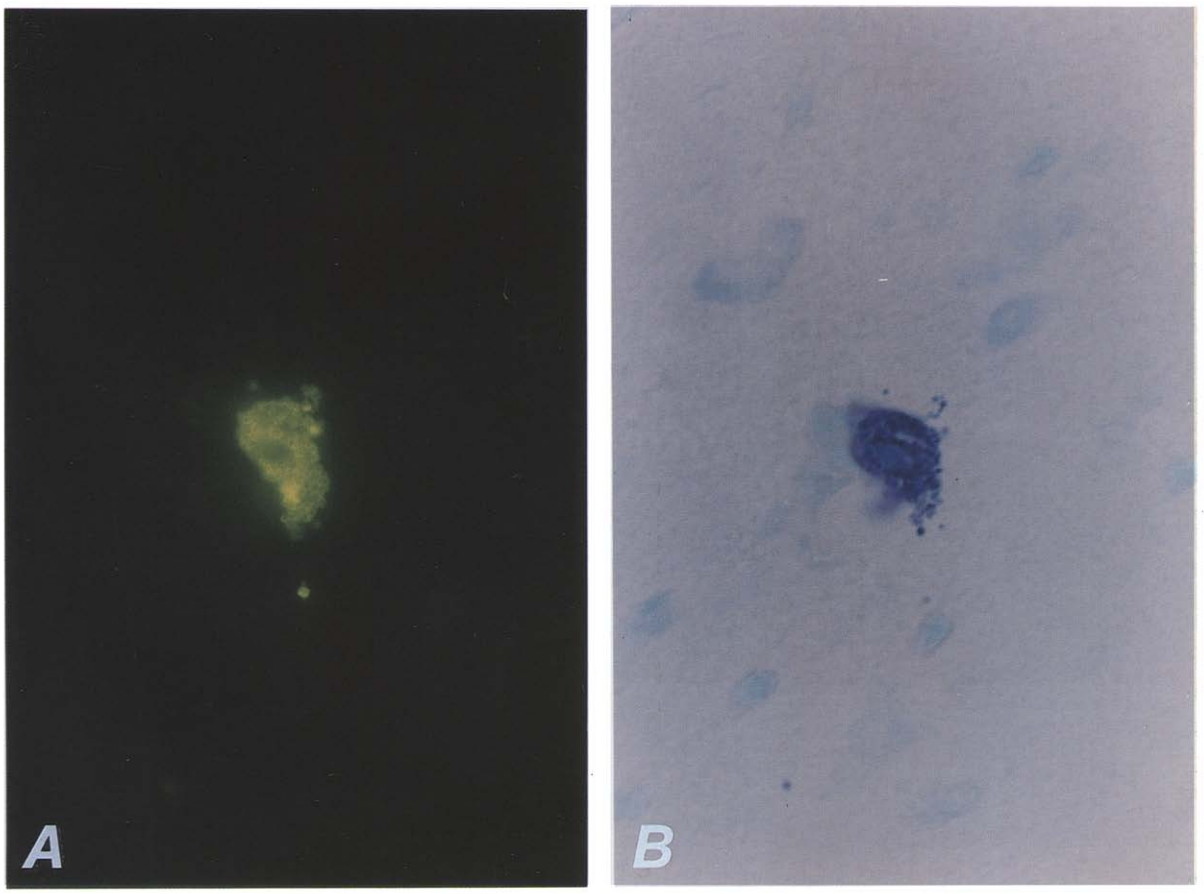


Fig. 5. (A) Photomicrograph showing immunofluorescence staining (streptavidin–FITC) for RMCP-1 in rat brain. (B) The same section counterstained with Toluidine Blue. Magnification: ×1000.

again and finally mounted in aqueous mounting medium. The specificity of this antibody was demonstrated by showing that it failed to label purified rat peritoneal mast cells after preabsorption with excess RMCP-I.

*Histamine.* Sections adjacent to those stained for RMCP-I were chosen for the demonstration of histamine using the avidin–biotinylated peroxidase complex (ABC) procedure. All specimens were treated with 0.3% $H_2O_2$ in methanol for 30 min to block endogenous peroxidase. After brief rinsing in PB, the sections were incubated in 5% normal goat serum in PB for 30 min, then exposed to rabbit polyclonal antibody produced against a histamine–bovine serum albumin (BSA) conjugate (Chemicon International) at 1:100 dilution for 1 h at room temperature. After three washes in PB, the sections were incubated with biotin-labeled anti-rabbit immunoglobulin G (ExtrAvidin Biotin Staining Kit, Sigma) at 1:20 dilution for 30 min at room temperature. The ABC reagent (Pierce) was applied to the sections for 45 min at room temperature. The peroxidase reaction incorporated 3,3′-diaminobenzidine as the chromagen. The sections were then rinsed in PB, dehydrated and coverslipped with Permount. Immunocytochemistry using rabbit antibody against histamine coupled to human serum albumin (made by the same company which prepared the antibody used here) was used previously to successfully label gastric mast cells[21]; when this antibody was preabsorbed with an excess of histamine before use, it failed to label purified rat peritoneal mast cells, as done previously.[32]

*Rat mast cell protease I and histamine.* After RMCP-I staining as above, the slides were incubated with antibody to histamine. Streptavidin–rhodamine was used at 1:200 dilution for 30 min instead of the ABC reagent. The slides were then mounted in aqueous mounting medium.

*Immunoglobulin E.* The slides were first incubated with rat immunoglobulin E (IgE; Zymed Laboratories, South San Francisco, CA, U.S.A.) at 1:300 dilution for 1 h at 37°C, then treated with 5% normal horse serum, followed by mouse monoclonal anti-rat IgE antibody (Zymed) at 1:100 dilution for 1 h at room temperature. Addition of horse anti-mouse immunoglobulin G–biotin and incubation with streptavidin–FITC were carried out as described above. The specificity of this antibody was also demonstrated by the fact that it failed to label purified peritoneal mast cells after preabsorption with excess IgE (Zymed).

*Immunoglobulin E binding protein.* This method used a mouse monoclonal antibody (mAB BC4, a gift from Dr R. P. Siraganian) which binds to rat FcεRI as long as the FcεRI is not occupied by IgE. After 30 min treatment with 5% normal horse serum, the samples were incubated with the anti-rat FcεRI antibody at 1:1000 dilution for 1 h at room temperature followed by three washes in PB. The slides were then exposed to horse anti-mouse immunoglobulin G–biotin at 1:200 dilution for 30 min. After three washes, the sections were incubated in streptavidin–rhodamine at 1:200 dilution for 30 min and then washed again. Finally, the slides were mounted in aqueous mounting medium (Biomeda). This antibody failed to stain purified rat peritoneal mast cells after the mast cells had been preincubated with 0.1 μg/ml rat IgE (Zymed) for 60 min at 37°C, to occupy all available FcεRI on the surface and washed with PB.

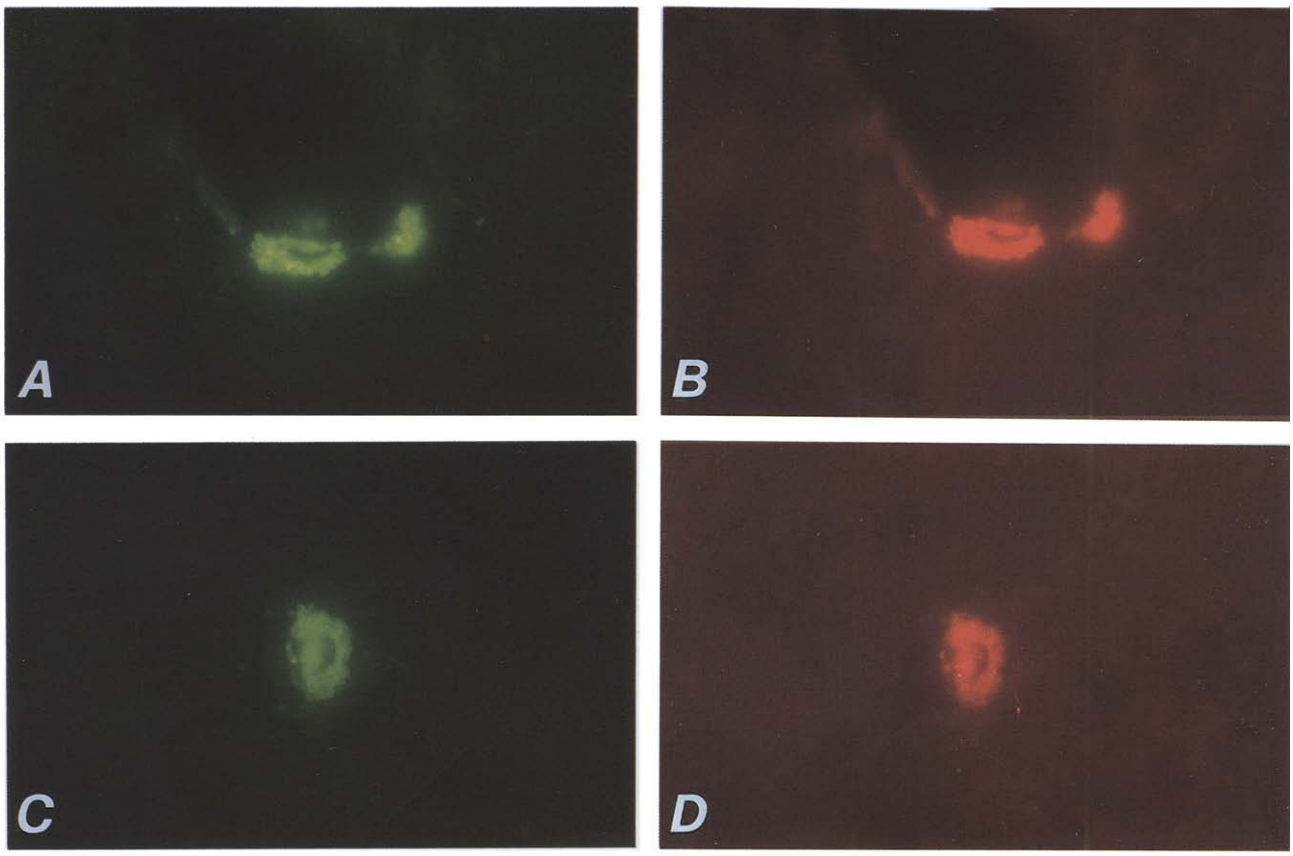


Fig. 6. Light micrographs of perivascular mast cells from the hypothalamus stained with fluorescein-labeled monoclonal mouse antibody to RMCP-I (A, C) and streptavidin–rhodamine-labeled polyclonal rabbit antibody to histamine (B, D). Magnification: ×1000.

In addition, in order to control for non-specific staining, the same immunohistochemical procedures described above were carried out on equivalent tissue sections, except that the primary antibodies were replaced with normal sheep serum, normal rabbit serum or phosphate-buffered saline.

*Toluidine Blue and berberine sulfate staining*

Samples were either counterstained with acidified (pH < 2.5) 0.5% Toluidine Blue or stained with 0.02% berberine sulfate for 20 min at pH 4, as described before.[54]

*Cell counting and photography*

Mast cells were counted in 400 continuous fields at ×200 (an area of 0.2948 $mm^2$) in six random sections of hypothalamus from each rat (total number of rats = 5) for RMCP-I and Toluidine Blue staining (Group No. 1), adjacent sections were stained for histamine and Toluidine Blue (Group No. 2). Mast cells are expressed as the mean number of cells/$mm^2$ counted ± standard deviation (S.D.), reflecting variability with the staining techniques used within each group, possibly as a result of sectioning.

*Electron microscopy*

Tissue blocks (1–2 $mm^3$) of hypothalamus, or the entire skull with the dura attached to it, were fixed by immersion in modified Karnovsky's fixative[27] containing 2% paraformaldehyde, 3% glutaraldehyde and 0.5% tannic acid in 0.1 M sodium cacodylate buffer for 10–12 h at 24°C followed by 12 h at 4°C. The tissue blocks, or the dura

Table 1. Hypothalamic mast cells stained immunohistochemically for rat mast cell protease I or histamine and counterstained with Toluidine Blue*

| | Group No. 1 | | Group No. 2 | |
|---|---|---|---|---|
| | RMCP-I | TB | Histamine | TB |
| Mast cells/rat† | 34 ± 7 | 34 ± 8 | 34 ± 6 | 34 ± 6 |

Mast cells/$mm^2$ = 0.3 ± 0.07.

*Each section in Group No. 1 was adjacent to the corresponding section in Group No. 2. TB, Toluidine Blue.

†Expressed as the mean number of mast cells from the five rats studied. The differences in S.D. reflect variability of the thickness of the section, permitting identification with the different stains.

carefully removed from the skull *en bloc*, were then washed in the cacodylate buffer, postfixed in 1% $OsO_4$ (Polysciences, Warrington, PA, U.S.A.) and washed twice in the cacodylate buffer. The samples were then dehydrated in a graded series of ethanol and embedded in Embed-812. One-micrometer-thick sections (1 $\mu$m) of Epoxy-embedded tissues were cut, stained with Toluidine Blue and evaluated by light microscopy. Thin sections (50–70 nm) were cut on an LKB 8801 ultramicrotome and stained with uranyl acetate and lead citrate (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Samples were prepared and photographed as described before.[54] Postembedding immunocytochemistry tissue was first etched with 10% $H_2O_2$.[37] This was followed by 1 h treatment with 10% normal donkey serum. After washing, experimental tissue was placed in primary antibody solution consisting of 1:50 dilution of sheep anti-rat RMCP-I antibody in Tris-buffered saline (TBS) with 1% BSA and 0.05% sodium azide (Moredun Animal Health, Edinburgh, U.K.). Control grids were placed in TBS alone. All grids were treated overnight at 4°C. Following one wash in TBS, all grids were treated with secondary antibody solution consisting of 1:15 donkey anti-sheep immunoglobulin G conjugated to 12 nm gold (Jackson Immuno Research Laboratory, West Grove, PA, U.S.A.). All grids were then treated with glutaraldehyde and $OsO_4$ followed by heavy metal staining. Grids were examined and photographed using a Phillips 300 transmission electron microscope.

## RESULTS

### *Histamine*

Histamine-containing perivascular hypothalamic cells were identified with immunohistochemistry using a polyclonal antibody against histamine–BSA (Fig. 1). Such cells exhibited distinct intracellular granular reaction deposits and typical morphological features of mast cells (Fig. 1A). The same antibody heavily stained purified homogeneic peritoneal mast cells used as positive control (Fig. 1B). Use of this antibody after it was preabsorbed with excess histamine did not stain the hypothalamus (Fig. 1C). However, use of the antibody without prior absorption identified numerous histamine-immunoreactive nerve fibers in the hypothalamus (Fig. 1D).

### *Heparin*

Staining with berberine sulfate showed that hypothalamic mast cells fluoresced brightly (Fig. 2), indicating the presence of heparin, which is typically found in CTMCs.

### *Immunoglobulin E binding protein mRNA*

The presence of Fc$\epsilon$RI mRNA was detected using the antisense probe in perivascular cells (Fig. 3A), which were identified as mast cells with Toluidine Blue (Fig. 3B). Hybridization with the sense probe did not detect any cells (Fig. 3C). The same antisense probe failed to show any signal in homogeneic cerebellum (Fig. 3D), which is known to be devoid of mast cells. Specificity of the antisense probe for Fc$\epsilon$RI mRNA was demonstrated by northern analysis using total RNA from RBL cells, which are known to express Fc$\epsilon$RI. The northern blot shows only one band where the nucleotides used hybridized, of 1 kb (Fig. 3E). This size is consistent with the Fc/RI cDNA, which is about 950 bp. Sense nucleotides did not react.

### *Immunoglobulin E and its binding protein*

Hypothalamic perivascular mast cells were negative for Fc$\epsilon$RI using a monoclonal antibody against Fc$\epsilon$RI (results not shown). Fluorescein-labeled mouse anti-rat IgE stained rat peritoneal mast cells strongly (Fig. 4A) and dura homogeneic mast cells weakly (Fig. 4B), used as positive control. It failed, however, to detect any hypothalamic cells with or without preincubation with exogenous rat IgE before use of anti-IgE (Fig. 4C).

### *Rat mast cell protease I*

Hypothalamic cells showed intense RMCP-I immunoreactivity with immunofluorescence (Fig. 5A). The same cells were also shown to have metachromatic granules when stained with acidified Toluidine Blue (Fig. 5B), which identifies them as mast cells.

### *Rat mast cell protease I and histamine*

Certain perivascular cells showed intense RMCP-I immunoreactivity with fluorescein (Fig. 6A, C). The same cells were also identified (rhodamine) for histamine with double immunohistochemistry (Fig. 6B, D), indicating that both mediators existed in the same perivascular cells.

### *Mast cell counts*

Toluidine Blue identified $34 \pm 6$ mast cells/rat. Of these, $34 \pm 7$ stained immunohistochemically for RMCP-I, while $34 \pm 6$ stained for histamine in adjacent hypothalamic sections (Table 1). The slight variability in the S.D. is probably due to differences in cell staining as a result of the sectioning.

### *Ultrastructure and immunogold labeling*

Transmission electron microscopy identified perivascular mast cells containing a typical nucleus with peripherally clumped cromatin, and numerous fairly homogeneous, intact, electron-dense granules (Fig. 7A). Other perivascular mast cells also contained numerous, smaller dense granules with lower electron density, as well as immature granules and possibly occasional lipid inclusions (Fig. 7B). Such cells were also shown to contain RMCP-I, as evidenced by the presence of gold particles strictly within their secretory granules when using postembedding immunogold labeling at the ultrastructural level (Fig. 8). The distribution of gold particles was the same in both dura (Fig. 8A) and homogeneic hypothalamic (Fig. 8B) mast cells, even though the latter contained somewhat fewer particles than the former, used as control.

## DISCUSSION

The present results confirm that the rat hypothalamus contains perivascular cells which store histamine, as demonstrated previously using histamine fluor-

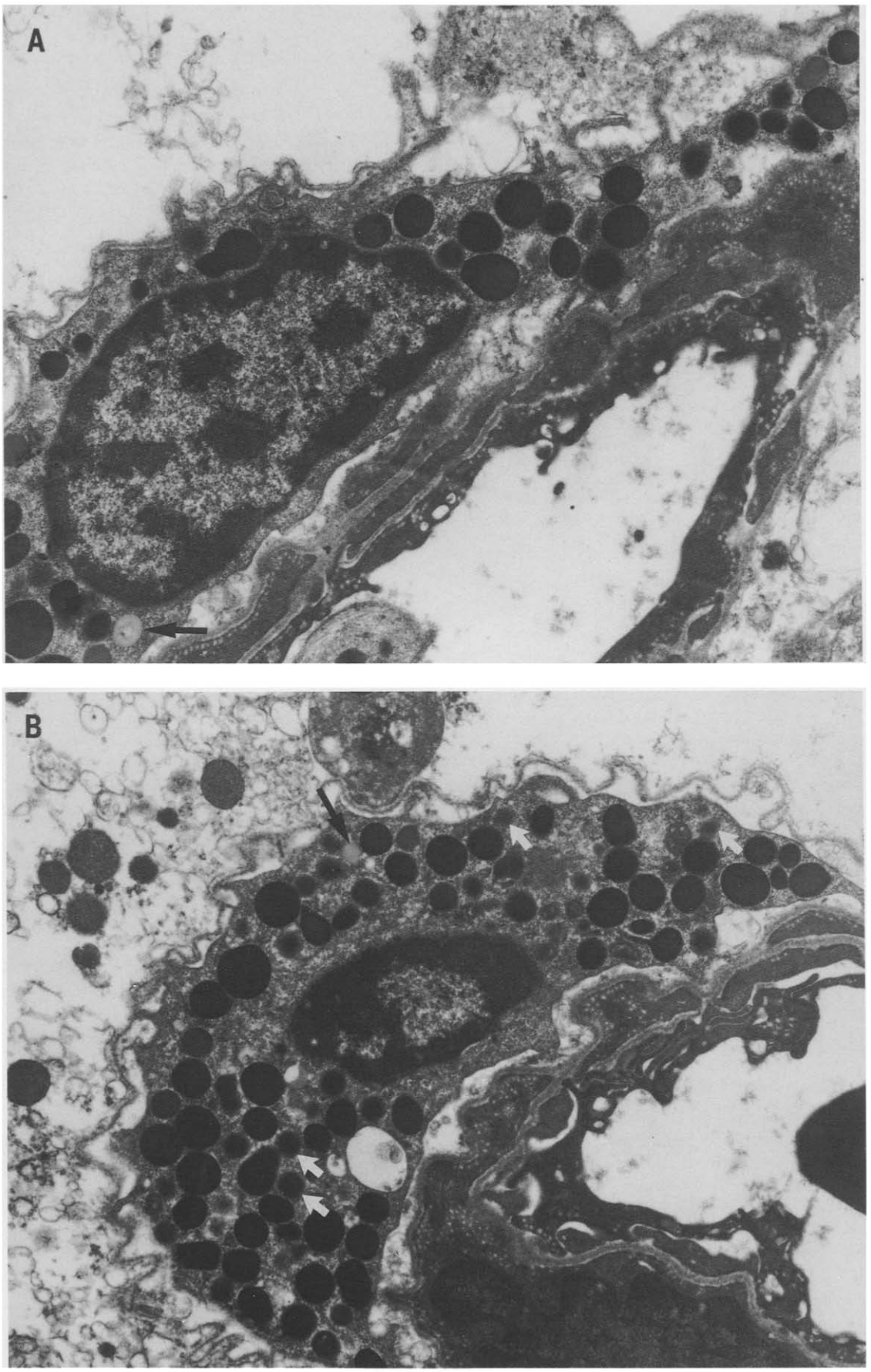


Fig. 7.

escence.[14] This is the first time, however, that these cells have been shown to also contain both heparin and RMCP-I, which characterize them as CTMCs. Moreover, our results using ISH showed that perivascular mast cells, recognizable with Toluidine Blue, had a very strong signal for FcεRI mRNA, which clearly establishes their mast cell nature. However, FcεRI was not detected in hypothalamic mast cells by either mouse anti-rat FcεRI or mouse anti-rat IgE, even though dura mast cells were weakly positive. This result suggests that either the amount of FcεRI expressed is below the level of our detection, or that under normal circumstances hypothalamic mast cells do not bind IgE, unlike their intracranial dura counterparts, which are outside the blood–brain barrier (BBB). During pathological conditions, however, IgE may enter the brain and could trigger mast cells to express FcεRI.

Mast cells had previously been shown to migrate into the thalamic area strictly along blood vessels from the choroid fissure and the fimbria of the hippocampus.[30] During this stage and until day 31 postnatally, BMCs were safranin-negative, indicating that they were either immature or MMC-like.[30] The time frame of mast cell migration coincided with neovascularization within the brain parenchyma, suggesting a possible reciprocal relationship in this development.[30] Mast cells from mixed slices of thalamus and hypothalamus were shown to secrete in response to C48/80 in a perfusion system[31] and this secretion was regulated by neuronal release of neuropeptides.[35] BMC secretion was also triggered *in vivo* by intracardiac administration of C48/80 and carbachol, while homogeneic peritoneal mast cells were not influenced by carbachol.[8] This finding differentiated BMCs from their extracranial counterparts. It was further shown that BMC secretion was accompanied by intragranular alterations of electron-dense material, and ultrastructural appearance which was distinct from the compound exocytosis commonly associated with CTMCs.[8]

Another unique aspect of the ultrastructure of activated BMCs is the presence of many empty-looking granules and a mixture of vesicles with various degrees of electron-dense content.[8] This appearance is clearly distinct from that of macrophages[8,26] and is quite similar to that reported for maturing rat BMCs,[25] or CTMCs,[5] human immature CTMCs[13] MMCs[15] or CTMCs "recovering" after secretion.[22] This unique appearance, and the presence of lipid inclusions in many activated BMCs,[8,26] has led investigators to describe such BMCs as neurolipomastoid.[26] Lipid inclusions may not be unrelated to BMC pathophysiology, since they have been associated with arachidonate metabolism or other fatty acid catabolism taken from the external environment,[12] and are particularly noticeable in immature mast cells.[13] However, there also exist some perivascular cells in the rat thalamus which apparently do not contain histamine, heparin or serotonin, and for which the term neurolipomastoid would not be appropriate.[9] Even though such cells have been reported to have characteristics of macrophages,[10] it remains to be shown that the same cells identified as neurolipomastoid at the ultrastructural level are also devoid of mast cell mediators using immunoelectron microscopy.

The distinct appearance of certain BMCs may be related to their unique neuronal microenvironment, even between intracranial mast cells outside and inside the BBB, since the former express FcεRI, while the latter do not appear to do so normally. Autofluorescent, Toluidine Blue-stainable Type II mast cells are located close to (20 nm) sympathetic, cholinergic and peptidergic varicosities in the cerebral vascular wall.[6] Antidromic stimulation of the trigeminal[7] or the superior cervical ganglion[28] could induce secretion from intracranial mast cells in the dura mater.[7] This effect, however, could be inhibited by activation only of intracranial presynaptic serotonin[4] and histamine[46] receptors, since extracranial (tongue) mast cells were unaffected. This is further indication that intracranial mast cells are pharmacologically distinct from their extracranial counterparts, even though they may have similar staining characteristics at the light microscopic level.

Recent reviews have stressed the possible significance of neuropeptide regulation of hypersensitivity reactions[16,41] and of interactions between mast cells and neurons.[50,57] The possible physiological role of BMCs still remains a mystery. Mast cells may regulate the permeability of the BBB and permit immune cells and pathogens[42,52] to enter. They may be responsible for vascular tone,[44] especially since they secrete vasoactive intestinal peptide,[34] which has been reported to be a major vasodilator in the cerebral vasodilator.[31] Hypothalamic mast cell activation may also be involved in mood and cognitive changes,[36] possibly associated with headaches.[51] In this context, it is interesting that BMCs were activated by immobilization stress,[49] they were localized close to corticotropin-releasing hormone-positive nerve fibers[55] and corticotropin-releasing hormone was shown to trigger skin mast cell degranulation at levels as low as 10 pM.[3] They may also participate in brain demyelination, which was recently shown to be triggered synergistically by estradiol and myelin basic protein.[53] For these reasons, mast cells have been implicated in multiple sclerosis,[29,52] a premise strengthened by the fact that the human

Fig. 7. Electron photomicrographs of perivascular mast cells from the hypothalamus. (A) Note multiple, homogeneous, intact, electron-dense granules. (B) Note numerous smaller granules with less electron density (white arrowheads) and possible lipid inclusions (solid arrows). Magnification for A and B: ×20,400.

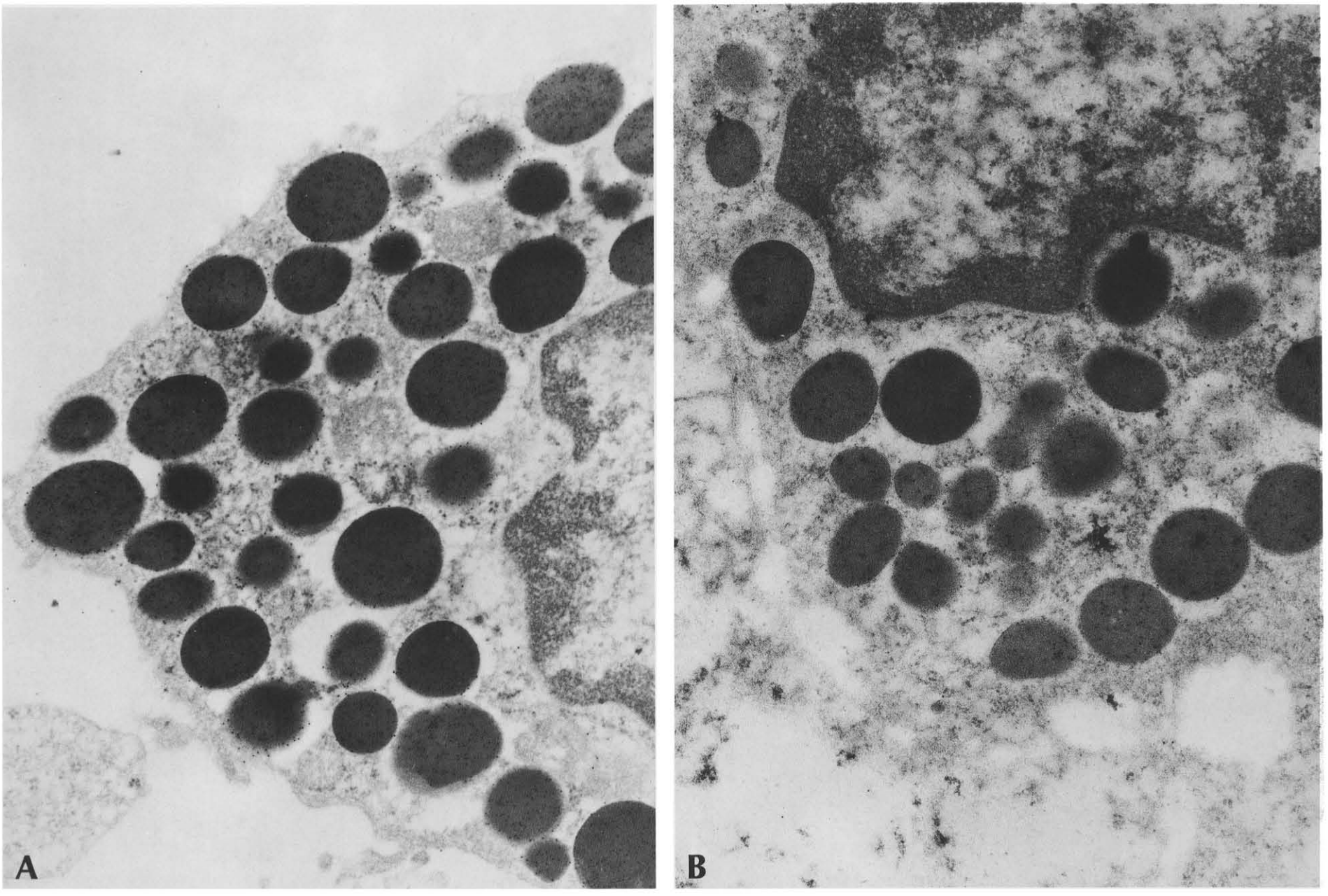


Fig. 8. Electron photomicrographs of portions of dura (A) and hypothalamic (B) mast cells showing secretory granules containing gold particles, indicating the presence of RMCP I

mast cell-specific enzyme tryptase was significantly elevated in the cerebrospinal fluid of multiple sclerosis patients.[45] BMCs may also be affected by female sex hormones, since CTMCs have been shown to express high-affinity receptors for estrogen,[39] and $\beta$-estradiol augments CTMC secretion in response to the neuropeptide substance P.[58] In fact, sexual courting after a period of isolation resulted in prompt appearance of BMCs containing gonadotropin-releasing hormone-like immunoreactivity in the habenula of male doves.[48] The BMCs may act as neuroimmunoendocrine master player, setting up the first response to stressful stimuli.

## CONCLUSIONS

Rat hypothalamic mast cells have all the ultrastructural and biochemical characteristics of CTMCs, such as secretory granule content of heparin, histamine and RMCP-I, as well as mRNA for Fc$\epsilon$RI, as shown by ISH. However, unlike intracranial CTMCs in the dura, which are outside the BBB and express some Fc$\epsilon$RI, hypothalamic mast cells do not appear to do so under normal circumstances.

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