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Research report

Morphological and functional demonstration of rat dura mater mast cell–neuron interactions in vitro and in vivo

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Abstract

Mast cells derive from a distinct bone marrow precursor and mature in tissues under the influence of stem cell factor, nerve growth factor (NGF) and certain interleukins. Intracranial mast cells first appear in the meninges and are located perivascularly close to neurons. They can be activated by antidromic stimulation of the trigeminal nerve, as well as by acute immobilization stress. Substance P (SP) and corticotropin-releasing hormone (CRH) are particularly potent in stimulating mast cell release of vasoactive, inflammatory and nociceptive molecules. These findings have suggested that mast cells may be involved in neuroinflammatory conditions, such as migraines. In this study, dura mast cells were shown to have characteristics of connective tissue mast cells (CTMC) as they contained histamine, heparin and rat mast cell protease I (RMCP-I). Mast cells were localized close to SP-positive neurons immunocytochemically and mast cell–neuron contacts were also documented using scanning electron microscopy. Dura stimulated by SP and carbachol in situ released histamine. Preincubation of dura with estradiol slightly augmented histamine release by SP, an effect possibly mediated through estrogen receptors identified on dura mast cells. Acute stress by immobilization led to dura mast cell degranulation which was prevented by pretreatment with a neutralizing antibody to CRH or a CRH receptor antagonist. The present results further clarify the biology of intracranial mast cells and support their involvement in the pathophysiology of migraines which are precipitated or worsened by stress.
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Keywords: Dura; Estradiol; Histamine; Mast cell; Migraine; Neuron; Neuropeptide; Secretion; Substance P; Stress

1. Introduction

A number of morphologic studies in different species have shown many perivascular mast cells in the leptomeninges, the choroid plexus, the thalamus, hypothalamus and the pineal [21,22,24,25,34,62,67]. Mast cells are especially plentiful in the dura and they contain a substantial proportion of total brain histamine [63]. Moreover, they are often found in close association with substance P (SP) and calcitonin gene related peptide (CGRP) containing neurons [39,45] that can in turn activate dura mast cells [18,64]. Vasoactive mediators and cytokines released from mast cells then increase vascular permeability and induce intercellular adhesion molecule (ICAM) expression [41,79]. Mast cell vasodilatory molecules such as histamine [42,47] nitric oxide (NO) [52], tumor necrosis factor (TNF) [29] and vasoactive intestinal peptide (VIP) [51] could participate in the vasodilatory phase of the migraine, which is associated with the throbbing pain. In fact, NO has been considered a key molecule in the pathophysiology of migraines [60]. Vasodilation was also documented by axial tomography during the acute phase of the migraine and

Abbreviations: CTMC, connective tissue mast cells; MMC, mucosal mast cells; C48/80, compound 48/80; IgE, immunoglobulin E; FcϵRI, IgE binding protein; NO, nitric oxide; PBS, phosphate-buffered saline; RMCP-I and -II, rat mast cell protease I and II; TNF, tumor necrosis factor; VIP, vasoactive intestinal peptide

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was prevented by the abortive antimigraine drug sumatriptan in humans [44].

There is no reliable animal model for migraines [23]. However, a substantial body of evidence indicates that activation of the trigeminal nerve leads to vasodilation and neurogenic inflammation [56], such as seen in humans during the migraine attack [59]. Moreover, activation of meningeal sensory neurons was recently proposed to be a primary mechanism in the origin of headaches [77]. Such trigeminal nerve-induced vasodilation and vascular permeability had been shown to depend on dura mast cell degranulation [16], while drugs clinically used for the symptomatic treatment of migraines inhibited trigeminal nerve stimulation-induced dura mast cell activation and vasodilation [6]. Additional evidence suggesting that dura mast cell activation is involved in human pathophysiology comes from biopsy studies showing that the temporal artery of the painful side of cluster headache patients contained degranulated mast cells [17,48,49]. Moreover, the serum level of the major mast cell mediator histamine in patients with migraine [33] and cluster headache [2] was increased indicating activation of mast cells, while histamine administration led to intense headache [42]. Most recently, we showed that migraine headaches in children are associated with urine elevations of the unique mast cell enzyme tryptase and that self-regulation by focusing on a relaxing image or piece of music for 10 min everyday, decreased the incidence of migraines and urine tryptase levels [61].

Migraines are known to worsen or precipitate with physical or emotional stress. In this respect, it is relevant that acute immobilization stress resulted in dura mast cell activation and increased rat mast cell protease I (RMCP-I) in the cerebrospinal fluid [85]. Moreover, we recently showed that corticotropin-releasing hormone (CRH), which is the first molecule to be released under stress, is a powerful trigger of mast cell activation and vasodilation [84]. A similar, but even more potent, action was documented for the CRH analog, urocortin [12]. Our hypothesis is that CRH released under stress activates trigeminal sensory afferent or sympathetic fibers, a premise supported by the fact that CRH-receptors were expressed on the sensory nuclei of the trigeminal nerve [69]. Neuropeptides secreted following orthodromic or antidromic stimulation can then trigger, alone or with CRH, dura mast cell release of vasodilatory, proinflammatory and neurosensitizing molecules that could contribute to the pathophysiology of migraines.

Here, we investigated the anatomical association between dura mast cells and neurons using light and electron microscopy, as well as any functional relationship between the two using acute immobilization stress of whole animals and in situ stimulation of dura mast cells. The results indicate that there are dura mast cell–neuron interactions that could have a functional significance and may help explain certain conditions such as migraine headaches.

2. Materials and methods

2.1. Tissue and cell preparation

Adult male Sprague–Dawley rats 250–300 g (Taconic, Germantown, NY) were anesthetized with an intraperitoneal injection of 0.5 ml ketamine/xylazine (20 mg/ml of each). The circulation was flushed with intracardiac administration of 50 ml normal saline (0.9% NaCl); the brain was then fixed by perfusion of 100–150 ml 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4 given also intracardially. After removing the brain, the skulls with the dura mater attached were postfixed in 4% paraformaldehyde at 4°C for 6 h. The dura mater was then carefully detached from the skull and kept in 4% paraformaldehyde at 4°C overnight. For berberine staining, the rat was perfused intracardially with 100–150 ml of a solution containing formaldehyde, acetic acid and methanol (FAM, 1:1:8) and then dissected from the skull and postfixed in FAM for 12 h. All tissues were then soaked in 20% sucrose in PBS at 4°C overnight, were frozen in Polyfreeze tissue freezing medium (Polysciences, Warrington, PA) and the frozen sections were cut at 7 μm with a cryostat (Jung CM 3000, Leica, Deerfield, IL). Sections were then stained and examined as described below. For electron microscopy [66], the dura mater was fixed with 2% paraformaldehyde, 30% glutaraldehyde and 0.5% tannic acid in 0.1 M Sorenson's buffer (pH 7.4).

Mast cells were obtained from the peritoneal cavity of the same rats from which the brain was also removed by lavage with HEPES-buffered Locke's solution containing (in mM): NaCl 150, KCl 5, $CaCl_2$, HEPES 5 and 1 g/l bovine serum albumin, 1 g/l dextrose (pH 7.2). Mast cells were then purified (purity > 90%) by centrifugation through 22.5% metrizamide (Accurate Biochemicals, Westbury, NY) at $350 \times g$ for 10 min; they were then washed three times in Locke's solution ($200 \times g$ for 5 min), fixed in 4% paraformaldehyde for 1 h, washed in 0.1 M PBS for 30 min, frozen and cut at 7 μm sections with a cryostat (as above).

2.2. Neonatal capsaicin pretreatment

Neonatal male Sprague–Dawley rats were injected subcutaneously in the first 2 days of life with 50 mg/kg of capsaicin (Polysciences) diluted in 0.05 ml of a solution containing 0.9% NaCl–100% ethanol–Tween 80 (8:1:1). Control animals received the same volume of vehicle. The animals were then returned to the cages and maintained (three per cage) on a 10:14 light:dark lighting cycle and allowed access to food and water ad libitum. They were used 4–6 weeks later when they reached 250–300 g of weight.

2.3. Immobilization stress

Male Sprague–Dawley rats, each weighing approximately 250–300 g (Taconic), were housed in plastic cages (three per cage) with a wire top in a modern animal facility under the supervision of veterinarians. They were allowed food and water ad libitum and were maintained in an automatic 14:10 h dark:light cycle. Animals were kept in the animal facility for 1 week before use. Each rat was brought into the laboratory one at a time between 0900–1100 h (to avoid any effect of diurnal rhythms) for 30 min every day for 5 days in order to accustom them to the stress of handling [8]. On the 6th day, each control rat was left in its cage for 30 min, while the experimental rat was stressed for 30 min by placing in a plexiglass immobilizer (Harvard Apparatus, Cambridge, MA) located on a bench top in the laboratory at room temperature [85]. No rat was ever present or in close proximity, while another was stressed or dissected.

2.4. Stimulation of dura mater mast cells with neuropeptides in situ

Rats were freshly killed with CO_2 inhalation and decapitated. The skull was opened with scissors with one cut from the foramen ovale towards the eyes. The vertex of the skull was then spread open, the brain was rapidly removed with a concave spatula in the cold and the foramen magnum was sealed with agarose to prevent leakage. The exposed cranial cavity with attached dura was filled with 2 ml of 95% CO_2:5% O_2-saturated Krebs–Ringer bicarbonate buffer (KRB) containing (in mM): NaCl 118.0, KCl 4.85, KH_2PO_4 1.15, $MgSO_4$ 1.15, $NaHCO_3$ 25.0, dextrose 11.1; pH 7.4 with or without $CaCl_2$ 2.5. Mast cells were then stimulated in situ with 10^{-5} M of SP (Bachem, CA), 10^{-5} M carbachol or 0.1 μg/ml compound 48/80 (C48/80, Sigma, St. Loius, MO). For investigation of the effect of estrogen, the dura was bathed in a solution containing 10^{-5} M 17β-estradiol for 30 min before addition of 10^{-5} M SP for another 30 min at 37°C. The volume of the bathing solution was measured at the end of the incubation to correct for any differences due to fluid absorption or leakage. One milliliter of supernatant from the cranial cavity was used for histamine determination with a radioenzymatic assay as described before [86]. Half of the dura was excised, freeze-thawed twice and was disrupted using a Polytron (Brinkmann Instruments, Westbury, NY), four pulses at maximum intensity for 15 s each at 4°C. The tissue was then centrifuged $3000 \times g$ for 30 min at 4°C and the supernatant was assayed for histamine. The second half of the dura was fixed for light and electron microscopy.

2.5. Toluidine blue and berberine sulfate staining

Dura mater samples were stained with acidified 0.5% toluidine blue (pH 2.5) for 5 min or with 0.02% berberine sulfate (pH 4) for 20 min, respectively, as described before [66] and were examined using a light microscope (Nikon, Don Santo, Natick, MA, USA) by two observers who were blind to the experimental conditions. Mast cell degranulation was defined as granule content visible outside the cell or loss of over 50% of cellular staining or both.

2.6. Cholinesterase staining

Dura mater fixed in situ with FAM for 2 h was removed and placed in a medium consisting of 2.5 ml maleate buffer (0.82% NaOH, 0.51% maleate, pH 6), 0.5 ml 3.5% sodium citrate, 1 ml 0.75% copper sulfate and 1 ml 0.165% potassium ferricyanide for 30 min at room temperature. This was followed by incubation in the same buffer with 0.05% acetylthiocholine (Sigma) for 18–19 h at 4°C. After a wash in the maleate buffer and dilute HCl (pH 2.5), the dura was stained with 0.5% toluidine blue (pH 2.5) for 5 min, followed by routine dehydration, cleaning and mounting. The tissue was examined and photographed under light microscopy as described before.

2.7. Electron microscopy

After immersion fixation of the dura in 2.5 glutaraldehyde and removal from the skull, the samples were washed in PBS, postfixed in 2% osmium tetroxide, dehydrated in graded ethanol solutions and embedded in Epon. Semi-thin sections (0.5 μm) were stained with toluidine blue, while ultrathin sections (300 A) were contrasted in 50% uranyl acetate in ethanol and 0.1% aqueous lead citrate. Grids with the sections were examined and photographed using a Joel 100S or a Philips 300 transmission electron microscope or an ISI-DS 130 scanning electron microscope (R.J. Lee, Trafford, PA) [15]. Degranulation was defined by the presence of granules with altered or absent electron dense content (transmission EM) or granules bulging from the cell surface, along with exocytotic cavities and loss of filopodia (scanning EM). Examination of the cells was carried out by two investigators blind to the experimental conditions.

2.8. Immunohistochemistry

2.8.1. Histamine

For demonstration of mast cell histamine, the fixed dura samples were subjected to the avidin biotinylated peroxidase complex (ABC) procedure. All sections were treated with 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase. After rinsing in PBS, the samples were incubated in 5% normal goat serum in PBS for 30 min, then exposed to rabbit anti-histamine polyclonal antibody (Chemicon International, Temecula, CA, USA) at 1:100 dilution for 1 h at room temperature. The sections were washed three times in PBS and then incubated with

biotin-labeled anti-rabbit IgG (ExtrAvidin Biotin Staining Kit, Sigma) at 1:20 dilution for 30 min at room temperature. The ABC reagent (Pierce, Rockford, IL, USA) was applied to the sections for 45 min at room temperature. The peroxidase reaction incorporated 3,3′-diaminobenzidine (DAB) as the chromagen. The sections were then rinsed in PBS, dehydrated and coverslipped with Permount.

2.8.2. RMCP-I and -II

Slides with frozen sections were brought to room temperature from −50°C for drying. All subsequent steps were also performed at room temperature. After the slides were rinsed in PBS, they were treated with 5% normal donkey serum and 5% normal horse serum for 30 min followed by incubation with sheep anti-RMCP-I polyclonal antibody or mouse anti-RMCP-II monoclonal antibody (Moredun Animal Health, Edinburgh, Scotland, UK) at 1:500 and 1:1000 dilution for 1 h, respectively. For secondary antibody, the sections were incubated with biotin conjugated donkey anti-sheep IgG (Chemicon) at 1:100 dilution (for RMCP-I) or horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) at 1:200 dilution (for RMCP-II) for 30 min. After three washes in PBS, the sections were incubated with streptavidin–FITC (Pierce) at 1:200 dilution for 30 min, they were washed again and finally mounted in aqueous mounting medium [66].

2.8.3. Estrogen receptor

Frozen sections of rat dura mater, and imprints of human breast tumor tissue made on histological slides as a positive control, were air dried for 5 min at 4°C and were treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase. The slides were then gradually dehydrated/rehydrated as follows: the specimens were washed for 5 min in 30%, 50%, 75%, 95%, and 100% ethanol followed by xylene and reversed process back to 30% ethanol. The sections were then washed out of residual ethanol by three 5-min washes in normal saline, followed by 30 min incubation with 5% normal goat serum at room temperature. After removing the excess normal goat serum from the slides, rabbit anti-human estrogen receptor polyclonal antibody (gift from Dr. Santhi Raam) was added to each slide at 1:60 dilution at 37°C for 30 min [65]. The slides were then washed three times in normal saline for 5 min. For secondary antibody, the samples were incubated with bi-

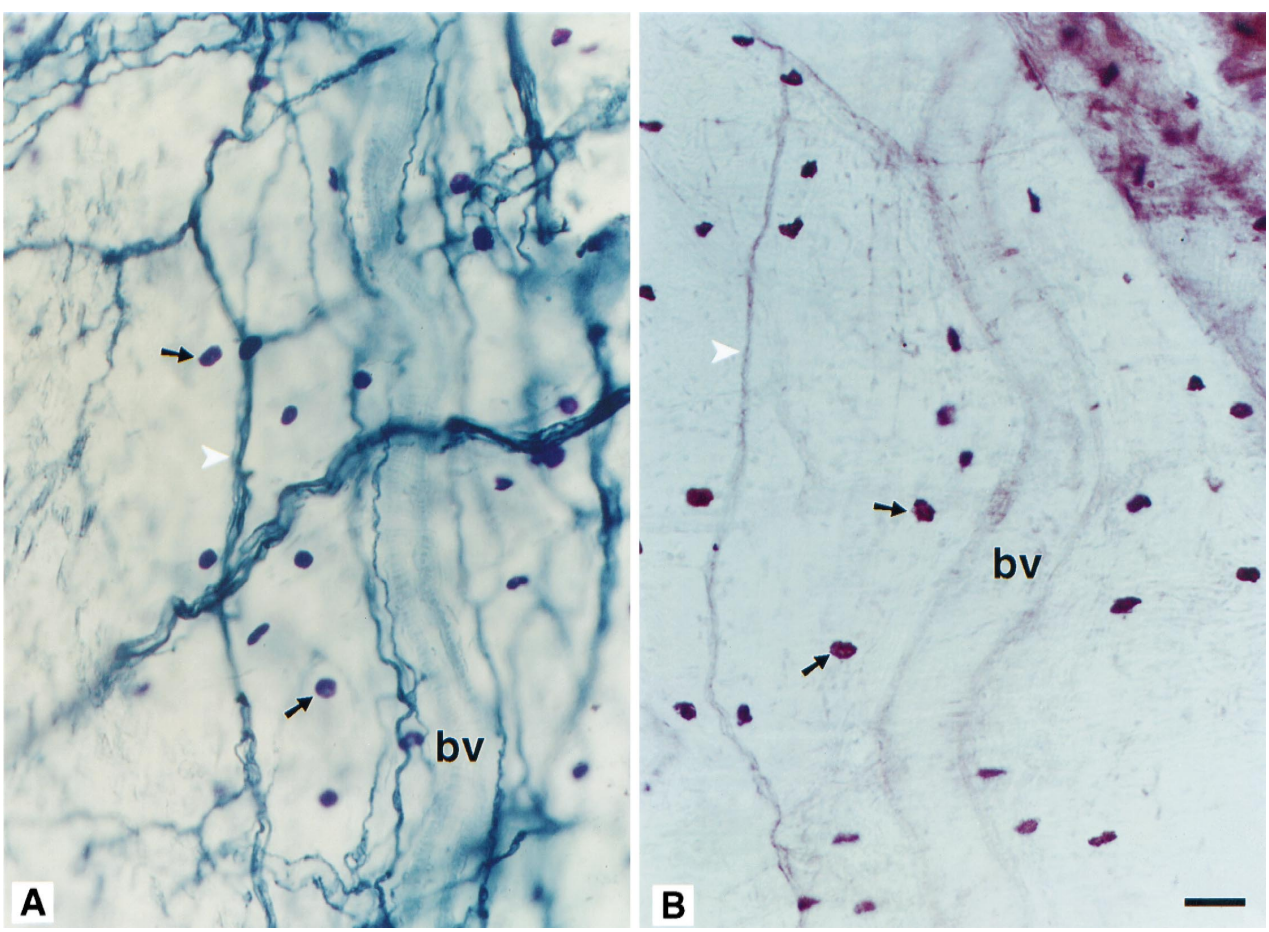

Fig. 1. Light photomicrographs of dura mast cells stained with either: (A) toluidine blue or (B) berberine sulfate; bv = blood vessel; white arrowhead = nerve fiber; black arrow = mast cell. Bar = 20 μm.

otin-labeled goat anti-rabbit IgG (Sigma) at 1:20 dilution for 30 min at room temperature. Following three washes in normal saline, the sections were incubated with streptavidin–horseradish peroxidase (Pierce) at 1:200 dilution for 30 min at room temperature. Visualization of the estrogen receptors was carried out by incubating the sections with 3′, 3′-diaminobenzidine (DAB)/H_2O_2. After dehydration and cleaning by gradual ethanol and xylene, the sections were mounted on the slides and cover-slipped with Permount. The sections were examined and photographed at 400 × before and after counterstaining with 0.5% toluidine blue (pH 2.5) for 30 min at room temperature, to visualize mast cells.

2.8.4. SP

Dura mater samples were fixed in 4% paraformaldehyde overnight than washed in 20% sucrose in 0.1 M PBS for 18 h in 4°C. Sections on the slides were washed with 0.1 M PBS for 5 min and then with 0.3% H_2O_2 in 100% methanol for 30 min in room temperature. Following three washes in PBS, sections were treated in 5% normal goat serum in 0.4% Triton X-100/PBS. They were then washed three more times and were treated with goat anti-rabbit IgG antibody (1:20 dilution) (Sigma) in room temperature for 30 min, following which the primary rabbit anti-SP polyclonal antibody was applied at 1:500 dilution (Chemicon) for 1 h at room temperature. After subsequent washes, streptavidin (1:200 dilution) was applied for 30 min in room temperature, followed by washes in PBS and DAB to develop color reaction. The slides were then rinsed in tap water, stained with 0.5% toluidine blue (pH 2.5) for 30 min and dehydrated in alcohols. After two 5-min washes, hemo-D was applied, coverslips were mounted on the slides and the sections were examined by light microscopy [66].

To control for non-specific staining, the respective procedures were carried out on equivalent sections, except that the primary antibodies were replaced with either normal sheep serum (for RMCP-I) or normal rabbit serum (for histamine, estrogen receptor and SP).

2.8.5. Statistical analysis

Results with cell counts or histamine measurements were analyzed and compared with the unpaired Student's *t*-test or with non-parametric analysis using the Mann–Whitney *U*-test. Significance is denoted by $p < 0.05$.

3. Results

3.1. Characteristics of dura mast cells

3.1.1. Toluidine blue staining

Dura mast cells fixed in situ were mostly elongated and contained many cytoplasmic granules that were stained intensely with toluidine blue (Fig. 1). The dura mast cells were often proximal to blood vessels and appeared dark blue (in thick sections) after staining with acidified toluidine blue (Fig. 1A) or brown-red after staining with berberine sulfate both of which identify the presence of heparin (Fig. 1B). Scanning electron micrographs showed mostly oval mast cells with prominent secretory granules, but few filopodia (Fig. 2); in contrast, purified homogeneic peritoneal mast cells were round with numerous filopodia (Fig. 2A, insert). Under transmission electron microscopy,

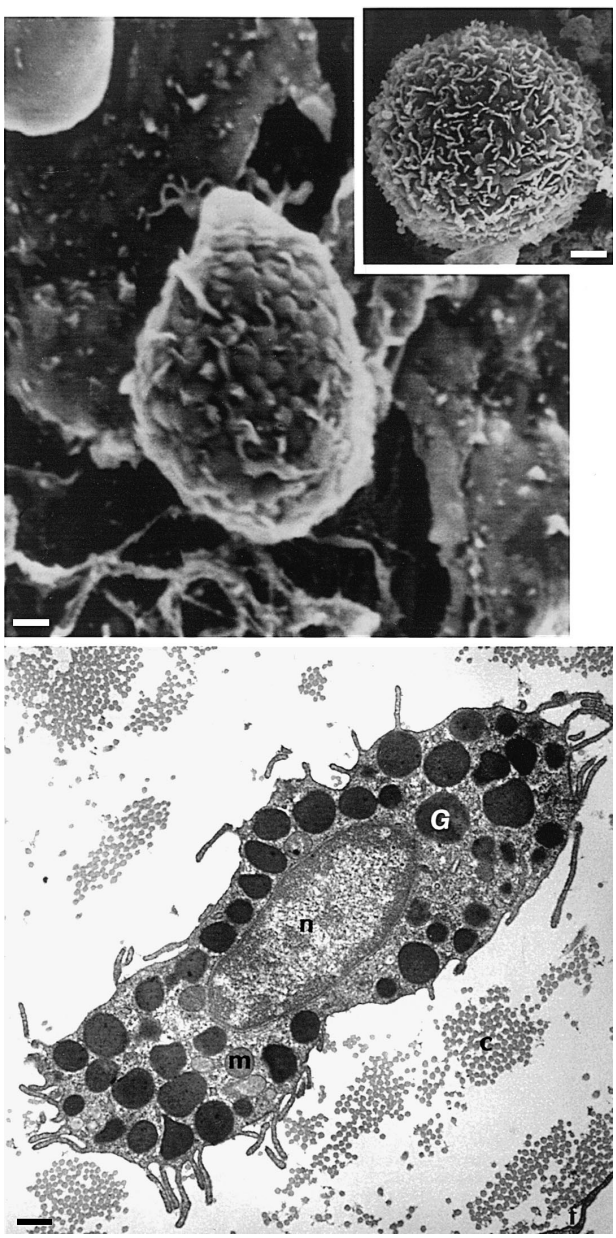

Fig. 2. Electron micrographs of control mast cells. (A) Scanning electron microscopic image of a dura mast cell with dense secretory granules and few filopodia; bar = 1.97 μm. Insert shows a homogeneic peritoneal mast cell with fewer granules close to the surface and numerous filopodia; bar = 1.41 μm. (B) Transmission electron microscopic image of a control dura mast cell showing intact secretory granules (G), a few mitochondira (m) and a prominent nucleus (n); bar = 0.13 μm.

mast cells contained numerous, round electron dense, spherical granules of uniform size. A few mitochondria, vesicles, Golgi apparatus and short filopodia were noted, along with an oval nucleus with peripherally clumped chromatin (Fig. 2B). Secretory granules were obvious, especially close to the surface of the cells, but they were tightly packed with no evidence of exocytosis (Fig. 2B). Scanning electron microscopy showed mast cells often ''wrapped'' around blood vessels (Fig. 3).

3.1.2. Neutral proteases

In addition to staining with toluidine blue and berberine sulfate, which bind to secretory granule heparin, dura mast cells were further characterized immunohistochemically for the presence of other secretory granule markers. Almost 70% of dura mast cells were positive for RMCP (brown)-I, while less than 10% stained for RMCP-II indicating that most were connective tissue mast cells (CTMC). All dura mast cells stained strongly yellow/green for histamine (results not shown).

3.1.3. Estrogen receptor

Certain dura mater cells stained weakly for the presence of estrogen receptor (Fig. 4A). When counterstained with toluidine blue, these cells were identified as mast cells

Fig. 3. Scanning electron micrographs of one dura mast cell wrapped around a capillary in each case. Bars = 7.58 μm.

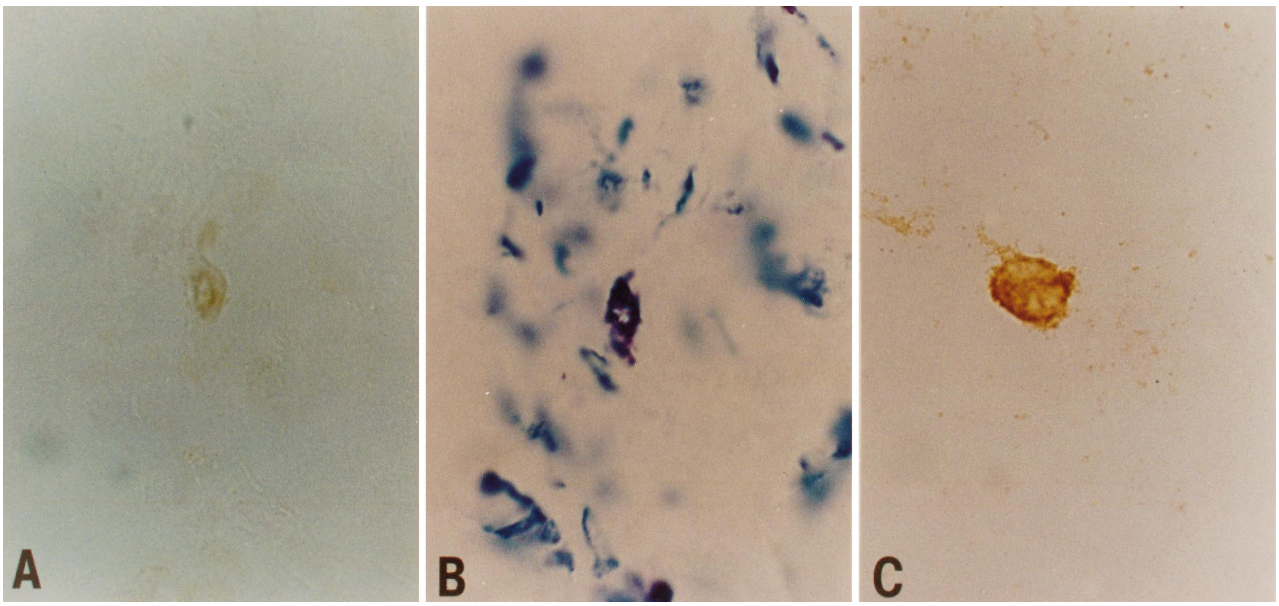

Fig. 4. Light micrographs showing dura cells: (A) stained immunohistochemically for estrogen receptor and (B) counterstained with toluidine blue to identify mast cells; (C) a breast cancer cell stained for estrogen receptor (positive control). Magnification = 1000 × .

(Fig. 4B). Human breast cancer cells used as control were strongly positive (Fig. 4C).

3.2. Mast cell–neuron associations

Staining of the dura mater for the presence of acetylcholinesterase (Fig. 5) showed a dense net of nerve fibers covering a substantial portion of the tissue area (Fig. 5A). Counterstaining with toluidine blue showed numerous mast cells lying within this network (Fig. 5B). On many occasions, mast cells were located in close proximity to neuronal processes (Fig. 6) and appeared as if they were touching (Fig. 6C, large arrowheads).

Direct ''contact'' with nerve fibers was quite apparent with scanning electron microscopy where characteristically granular mast cells were seen touching or being wrapped around neuronal processes (Fig. 7A–D).

3.3. Stimulation with SP in situ

Staining for SP showed numerous neuronal fibers positive for this neuropeptide often in the vicinity of capillaries (Fig. 8). Counterstaining with toluidine blue revealed mast cells, located perivascularly in close association to SP-reactive neuronal processes (Fig. 8).

Unstimulated mast cells were intact and stained intensely with acidified toluidine blue (Fig. 9A). In situ incubation of dura with 10^{-4} M SP resulted in degranulation of numerous mast cells recognized by the extensive loss of toluidine blue staining (Fig. 9B). A similar, but not as dramatic an effect, was seen when mast cells were activated by 0.1 μg/ml C48/80; in this case, the content of numerous secretory granules could be seen outside the cells that still stained strongly with toluidine blue (Fig. 9C). Electron microscopy documented mast cell degranulation as numerous secretory granules contained altered content or had obviously lost their electron dense content (Fig. 10A and B). On scanning electron microscopy, secretory granules of stimulated mast cells were now obvious on the surface of mast cells with large exocytotic pits apparent below (Fig. 10C and D).

Stimulation of dura mast cells was carried out in the absence of calcium ions in order to avoid the possibility of stimulating any histaminergic neurons. C48/80 and SP are known to trigger mast cell secretion in the absence of extracellular calcium ions. Histamine release from dura mast cells in situ was $10 \pm 8\%$ of total ($n = 6$) in the control, unstimulated tissue, while SP caused $25 \pm 9\%$ ($n = 5$) of total histamine release (Table 1). Carbachol at 10^{-5} M increased ($p < 0.05$) histamine release ($n = 4$) to $23 \pm 8\%$ of total (Table 1). Preincubation of dura with 10^{-6} M 17β-estradiol for 30 min released $15 \pm 7\%$ histamine ($n = 5$) that was not different from control ($p > 0.05$). However, 10^{-6} M estradiol followed by 10^{-5} M SP increased ($n = 5$, $p = 0.05$) histamine release from $15 \pm 7\%$ to $37 \pm 9\%$ of total ($p = 0.05$) when compared to SP alone (Table 2). Neonatal capsaicin treatment did not affect the ability of dura mast cells to respond to these agents (results not shown).

3.4. Acute immobilization stress

Immobilization of rats for 30 min led to degranulation of $68 \pm 8\%$ mast cells ($n = 5$) as compared ($p < 0.05$) to $36 \pm 8\%$ ($n = 4$) found in control rats (Table 2). This effect was reduced to $42 \pm 8\%$ ($n = 3$) in rats pretreated

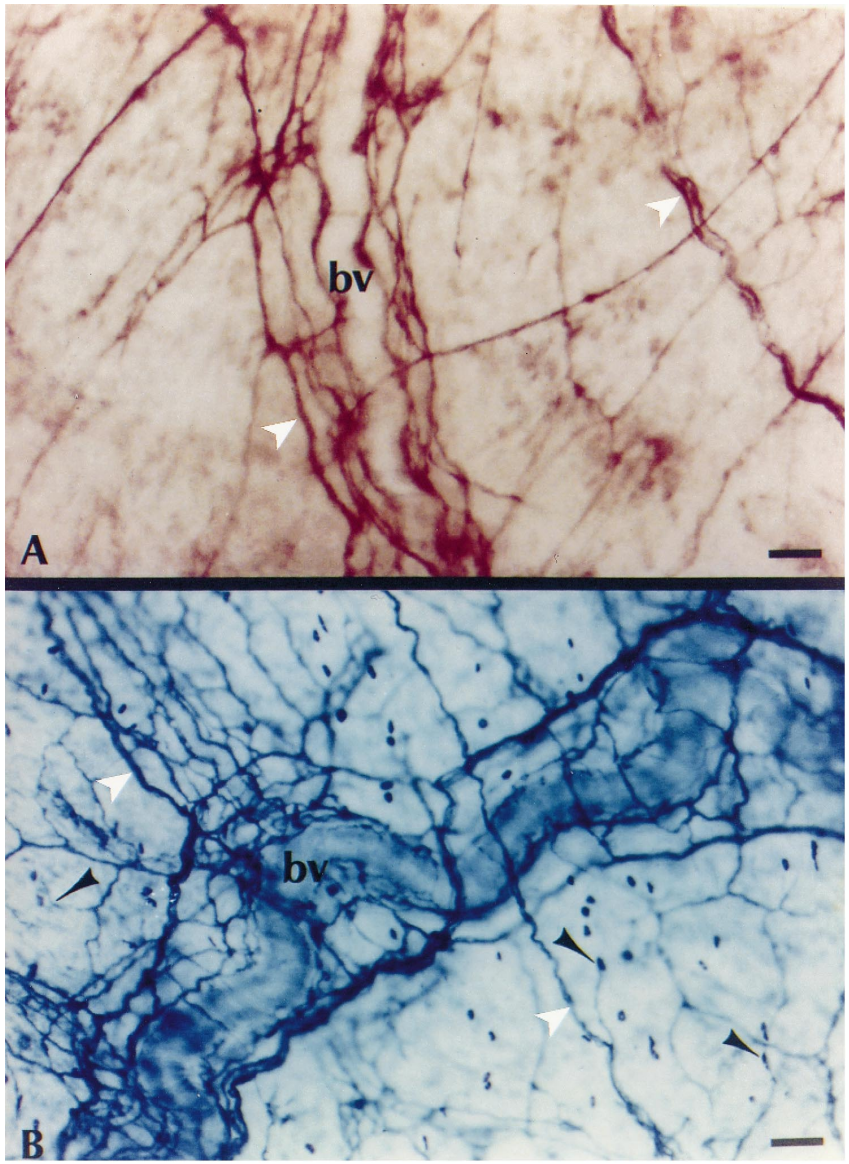

Fig.5

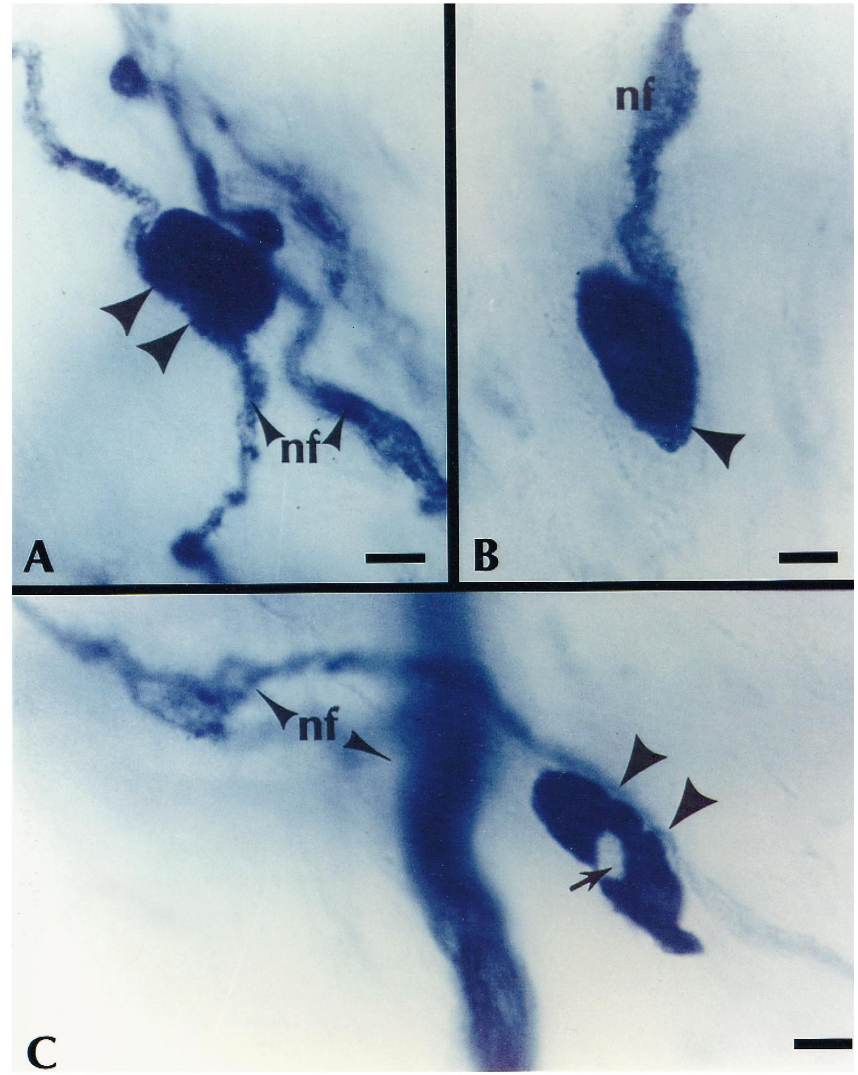

Fig.6

Fig. 5. Light photomicrographs of dura mater stained (A) for cholinesterase and (B) with toluidine blue. Note numerous nerve fibers (white arrowhead) and mast cells (black arrowheads); bv = blood vessel. Bar = 40 μm.

Fig. 6. Light photomicrographs of dura mater stained with toluidine blue (A, B, C) showing mast cells located close to neuronal processes (small arrowheads). Large arrowhead indicates points of presumed contact between nerve fibers and mast cells; small arrow denotes the nucleus; nf = nerve fiber. Bar = 4 μm.

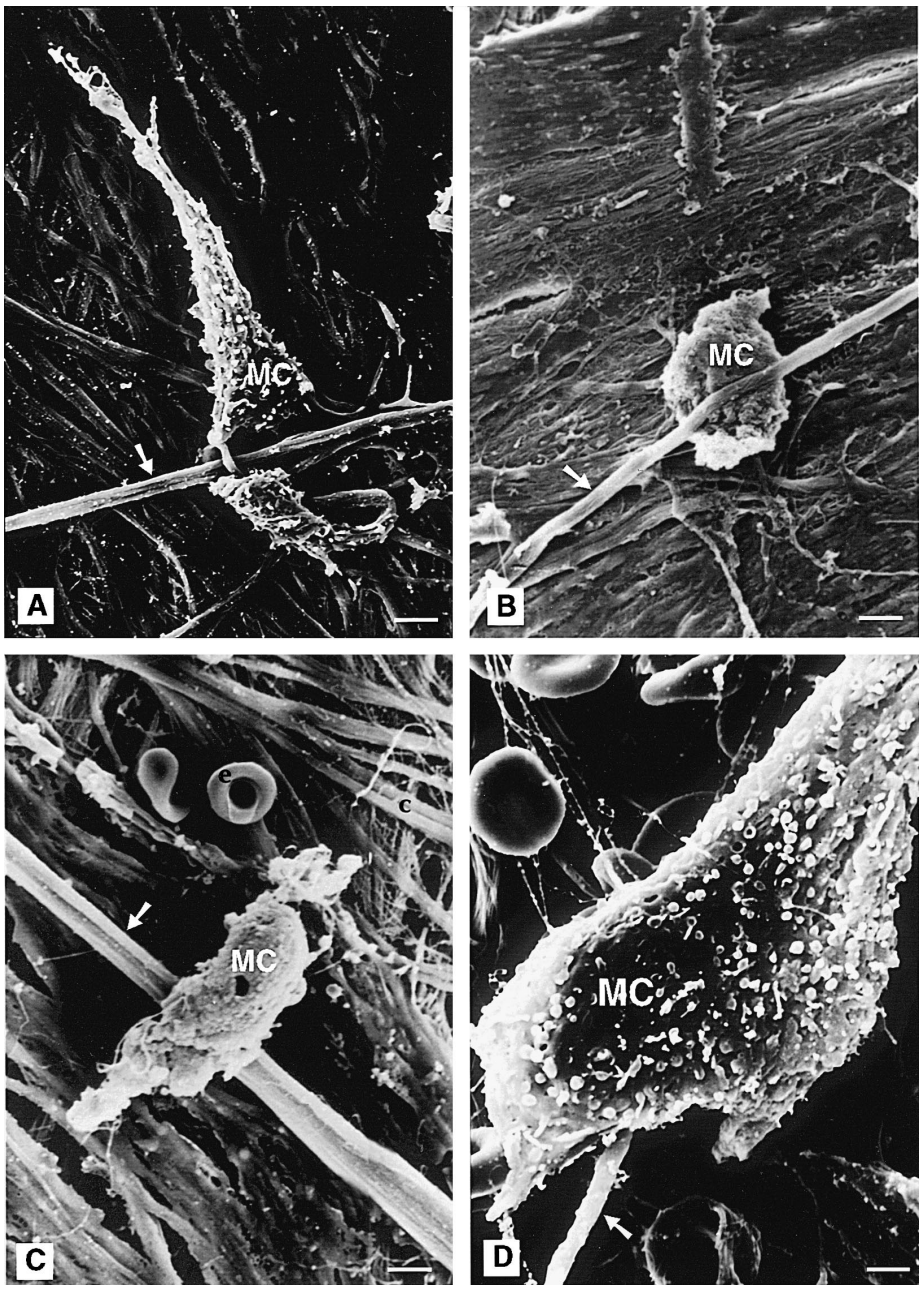

Fig. 7. Scanning electron micrographs of mast cells in close contact with neuronal fibers (white arrow); e = erythrocyte; c = collagen. Bars: A = 3.79 μm; B = 5.68 μm; C = 1.95 μm; D = 1.12 μm.

with the CRH receptor antagonist antalarmin administered i.v. 6 h prior to stress. Mast cell degranulation was also reduced to $35 \pm 8\%$ ($p < 0.05$) by a polyclonal anti-CRH serum, while normal rabbit serum did not appreciably affect ($p > 0.05$) mast cell degranulation that remained at $54 \pm 12\%$ (Table 2).

4. Discussion

The present data provide evidence for anatomical and functional interactions of dura mast cells with cholinergic and peptidergic neurons. Dura mast cells resemble CTMC since they were positive immunohistochemically for histamine and for RMCP-I, while they stained with toluidine blue and with berberine sulfate, both of which bind to heparin. These findings confirm previous reports with toluidine blue [66] or RMCP-staining [20]. Mast cells were seen in direct contact with neuronal processes, apparently containing SP. Peptidergic innervation of dura is well established [39] and close anatomical associations of mast cells with neurons were independently noted in the dura [15,19,39], as well as in the hypothalamus, [66] dermis [57] and mesentery [73,76]. Release of SP or CGRP from

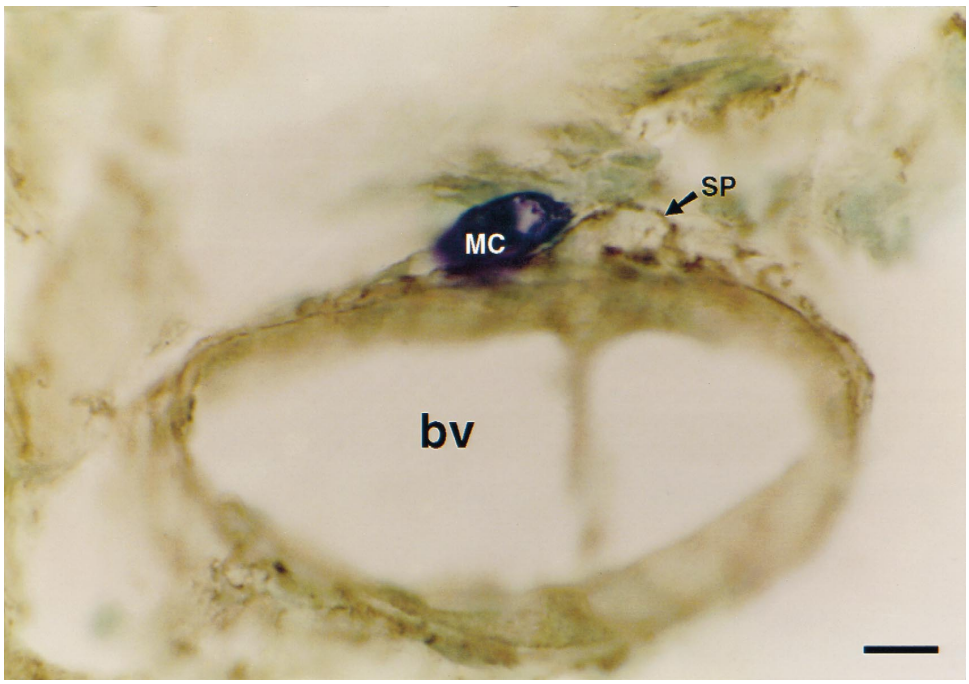

Fig. 8. Light micrograph of a dura mater stained immunohistochemically for SP and counterstained with toluidine blue, showing a dura mast cell (blue) adjacent to a blood vessel (bv) and to SP-reactive neuronal processes (brown). Bar = 5 μm.

sensory afferents could, therefore, stimulate dura mast cell secretion [35,82] as shown here and previously [18,64].

Rat dura mast cells were shown for the first time to weakly express high affinity estrogen receptors, a finding supported by the apparent increased histamine release in response to SP after preincubation with β-17 estradiol. We had previously demonstrated the presence of estrogen receptors on human bladder and human leukemic mast cells [65]. Moreover, we had shown that estradiol augments degranulation of mast cells by 48/80 [89], as well as myelin basic protein (MBP) or carbachol [83]. The observed release of histamine could not have come from histaminergic neurons as none were noted in the dura, as also reported by others [5]. Moreover, stimulation of dura mast cells was carried out in the absence of extracellular calcium to avoid stimulating any undetected histaminergic neurons. C48/80 and SP are known to stimulate histamine release from mast cells in the absence of extracellular calcium ions [46]. Receptor mediated release of histamine had previously been shown in response to SP and CGRP [64]. These findings may be relevant to mast cell involvement in the pathophysiology of migraines [80] and may help explain the higher prevalence of migraines in women or the frequent occurrence of migraines during ovulation.

Mast cells derive from the bone marrow [70] from where they migrate into different tissues and ''mature'' depending on the microenvironmental conditions present [29]. Mast cells are located perivascularly and in many cases in close proximity to neurons [3,14,58]. CTMC found in the skin and mucosal mast cells (MMC) vary considerably in their staining characteristics and their cytokine content [4,29]. Moreover, while CTMC can be readily activated by neuropeptides [28] such as SP, [1]. MMC are weakly responsive only to SP [35,50,71].

Mast cells contain and release numerous vasodilatory, proinflammatory and neurosensitizing molecules [29,82] such as histamine, TNF, VIP and NO. VIP [31,51] and NO [52,60] have also been implicated in headache. Moreover, mast cell activation by SP [26,32,64] led to granulocyte infiltration [54], possibly through TNF which can induce expression of endothelial adhesion molecule-1 [90]. SP is further involved in neurogenic inflammation [30] and in the transmission of pain, as shown with the use of NK-1 receptor knock-out mice [13]. The premise that SP stimulates mast cells to release vasodilatory molecules is supported by the fact that the anti-migraine drug sumatriptan blocked dura mast cell degranulation induced by trigeminal stimulation [7] and the newer analog rizatriptan blocked similarly induced vasodilation and fluid extravasation [92]. Our present findings support the contention that mast cells play a role in the pathogenesis of migraines [80]. Mast cell activation by parasympathetic and sensory neurons could lead to neurogenic inflammation [16], during which mast cell-derived molecules could lead to sensitization of meningeal sensory neurons and subsequent headaches [77]. In fact, serum histamine is elevated during migraine [33] and cluster headaches [2], the latter of which has also been correlated with ipsilateral temporal artery mast cell degranulation [17,48].

There are no reliable models for the study of headache [23]. In an attempt to mimic psychological stress known to precipitate vascular headaches, rats were subjected to a

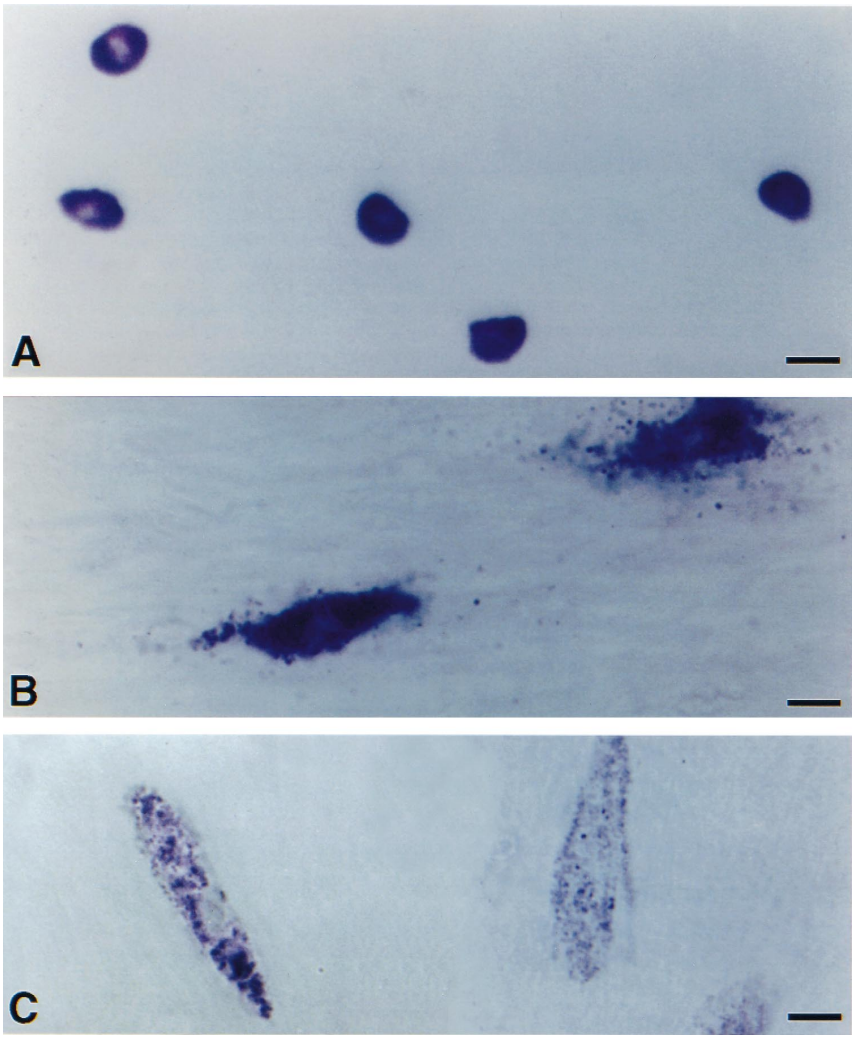

Fig. 9. Light photomicrographs of dura mast cells stained with toluidine blue following stimulation in the absence of extracellular calcium ions. (A) Control; (B) in response to 0.1 μg/ml C48/80; note granule contents outside cells; bar = 4 μm; (C) in response to 10^{-5} M SP; note loss of secretory granule content and staining; bar = 7 μm.

brief period of immobilization. This type of stress led to considerable dura mast cell degranulation which was blocked by pretreatment with a neutralizing antiserum for CRH, as well as by pretreatment neonatally with capsaicin, indicating an apparent involvement of sensory neuropeptides in vivo [84,85]. Here, we showed that dura mast cell degranulation by immobilization stress was inhibited by the non-peptide CRH-receptor antagonist antalarmin, clearly implicating CRH which can directly induce mast cell degranulation and vascular permeability in the skin [84]. These latter actions were mast cell dependent and may be mediated via CRH receptors since leukemic mast cells expressed CRH_1 receptors [84]. A direct in vivo action of CRH on vessels cannot be precluded since a CRH receptor subtype was recently identified on brain arterioles [9], but it is rather unlikely since the vasodilatory effect of CRH was absent in mast cell deficient mice [84], unless they also have some yet unrecognized defect in a putative endothelial CRH receptor. Our findings with dura mast cells could also be explained by an indirect effect of CRH through neuropeptide release since CRH receptor expression was shown on the principal sensory nuclei of the trigeminal nerve [69].

CRH is a 41 amino acid peptide is the principal regulator of the hypothalamic–pituitary–adrenal axis, thus coordinating the stress response [75] through specific receptors [10]. CRH administration to humans or animals causes significant peripheral vasodilation manifested as flushing, often accompanied by itching, symptoms consistent with mast cell activation [43,88]. Recent evidence suggests that CRH secreted outside the central nervous system (immunoreactive CRH, iCRH) has pro-inflammatory actions [11] in such models as carrageenin-induced aseptic subcutaneous inflammation [37]. Both iCRH and CRH mRNA have also been demonstrated in leukocytes while CRH also stimulates lymphocyte proliferation, increases IL-2 receptor expression on T-lymphocytes and is chemotactic for mononuclear leukocytes [36]. The demonstration of CRH-

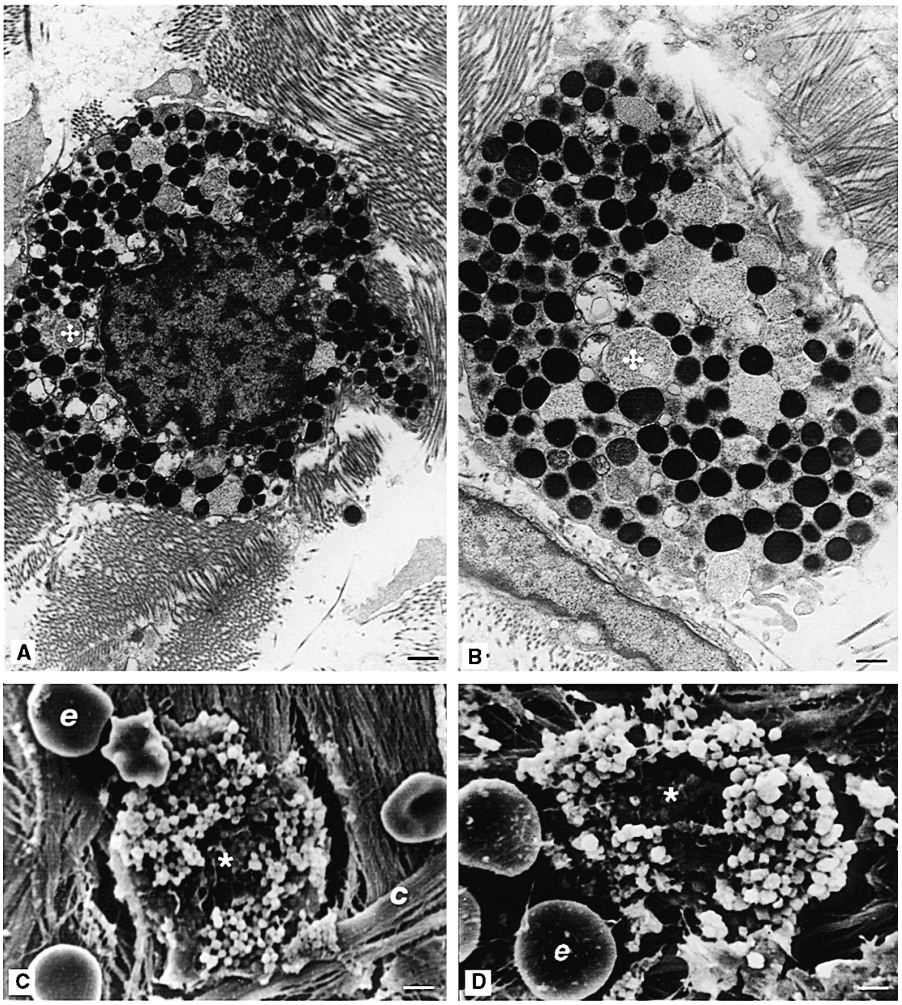

Fig. 10. Transmission electron micrographs of dura mast cells stimulated by (A) 0.1 μg/ml C48/80 or (B) 10^{-5} M SP; note granules that have lost their electron density (white cross). Scanning electron micrographs of dura mast cells degranulated stimulated by (C) 0.1 μg/ml C48/80 or (D) 10^{-5} M SP. Bars: A = 1.67 μm; B = 1.23 μm; C = 2.46 μm; D = 1.83 μm.

like immunoreactivity in the dorsal horn of the spinal cord and dorsal root ganglia [55,73,74], as well as in sympathetic nerve cell bodies and sympathetic ganglia [55,78,87], implies that antidromic or orthodromic nerve activation could lead to release of CRH and subsequent mast cell stimulation. For instance, dura mast cells degranulated in response to antidromic trigeminal [15] or cervical [38] ganglion stimulation. These findings have raised speculations that mast cell–neuronal interactions [91], may be

Table 1
Histamine release from dura mast cells in situ
Supernatant and one excised dura half were assayed for histamine release. Histamine (ng) present in the supernatant and dura was determined from a standard curve. Detectable histamine release was expressed as percent of total, which was calculated as the histamine (ng) present in the supernatant over total histamine (ng)×100.

Condition	*n*	Histamine release (% total)
Control	6	10±8
Carbachol (10^{-5} M)	4	23±8*
SP (10^{-5} M)	5	25±9*
Estradiol (10^{-6} M)	5	15±7
Estradiol + SP	5	37±9*

* $p < 0.05$.

Table 2
Effect of immobilization stress on dura mast cells

Conditions	*n*	No. of mast cells studied	Degranulation (%)
Control	4	1326	36±8
Stress[a]	5	2660	68±8[b]
Anti-CRH + stress	3	763	35±8[c]
NRS + stress	4	1478	54±12
Antalarmin + stress	3	590	42±8[c]

NRS = normal rabbit serum.
[a] By immobilization for 30 min.
[b] $p < 0.05$ compared to control.
[c] $p < 0.05$ compared to stress.
n = number of animals.

involved in neuroinflammatory disorders [27,53,81,82] and in central nervous system pathophysiology [40,68,72].

The present results provide additional evidence in support of the premise that mast cells are responsible for regulation cerebral vasodilation and may mediate vasodilation during migraine headaches [80]. This hypothesis is further supported by the recent demonstration that the unique mast cell enzyme tryptase was elevated in the urine of children during a migraine attack and was reduced following training in self-regulation by focusing on a relaxing image or piece of music for 10 min everyday [61].

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