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City University of New York PH.D. 1985

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RAT BRAIN MAST CELLS: CHARACTERIZATION, QUANTIFICATION, AND RELATIONSHIP TO BRAIN HISTAMINE

by

Robert Goldschmidt

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Graduate School of the City University of New York

1985

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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date

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Abstract

The histological, histochemical and biochemical properties of mast cells (MCs) in rat brain were investigated. Brain MCs stained with aqueous toluidine blue (pH 2.3) and Astrablau (pH 1), and were morphologically similar to tongue MCs. Brain MCs also fluoresced after exposure to gaseous o-phthalaldehyde, indicating the presence of histamine (HA).

Brain MCs were almost exclusively (98%) within thalamus, where they exhibited a striking anterior-posterior distribution and large variation in number (210-31,930 MCs per thalamus). Thalamic MC numbers were significantly greater in females than in males ($P<0.02$) and in left hemisphere than in right ($P<0.005$).

MC numbers, HA levels and tele-methylhistamine levels (tMH, a HA metabolite) were analyzed from the same brain tissue. Thalamic MC numbers were highly correlated with both the amount (ng) and the concentration (ng/g) of thalamic HA in both sexes ($P<0.0005$). Slopes of these regression lines, suggestive of the HA content of thalamic MCs, were 2.5 and 1.3 pg/cell in males and females, respectively, substantially less than the HA levels in peritoneal MCs. Thalamic MC numbers were not correlated with HA (ng) outside of thalamus, but were significantly ($P<0.003$) correlated with whole brain HA amounts (ng) and levels (ng/g). These correlations indicate that thalamic MCs contribute up to 90% of the HA in

thalamus, and up to 50% of whole brain HA levels. Freezing of brain tissue before dissection (necessary to estimate MC numbers and HA levels in the same brain) had no effect on regional HA levels.

Thalamic MC numbers were significantly correlated ($P<0.01$) with the amount (ng) of tMH in thalamus, but not with thalamic tMH concentrations (ng/g). The slopes of the regression lines suggest that brain MCs contain 0.1 pg tMH per cell in both sexes, about 5% of the HA content. However, partial correlation analysis indicates that thalamic tMH levels are not directly related to MC numbers, but rather are related to thalamic non-MC HA levels. Thalamic MC numbers were not correlated with non-thalamic or whole brain tMH.

These results indicate that rat brain MCs are histologically similar to peripheral MCs, and that these cells contribute directly to HA but not tMH levels in brain.

Acknowledgements: I would like, foremost, to thank my teacher and advisor, Dr. Lindsay Hough. Without his help, this work would not have been possible. I would further like to thank Drs. Stanley Glick and Jacques Padawer for their interest and superb input.

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Finally, I would like to thank my wife Michele, whose love, support and patience allowed me to get through the difficult times encountered during these studies. Upon her I confer the degree of Ph.T. (Putting her husband Through).

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Mast cells (MCs), first described by Paul Ehrlich (1877, 1879), have been of interest to a diverse group of researchers for both anatomical and physiological reasons. They have been implicated in a number of pathological syndromes and are hypothesized to function in several "normal" physiological roles (see Introduction section I-I). In addition to being present in a wide range of species (Selye, 1965), MCs constitute a broad spectrum of cells that share certain characteristics, requiring the use of an operational rather than an inherent definition: "a MC is a connective tissue element which possesses cytoplasmic granules that stain metachromatically under ordinary conditions" (Selye, 1965).

The evidence for histamine (HA) having a neurotransmitter function in the central nervous system (CNS)(see review by Hough and Green, 1984a) has led to renewed interest in the possible presence and function of MCs in brain. As peripheral MCs contain several substances (including HA) that have potent neuronal and non-neuronal activities, the hypotheses that MCs a) exist in brain b) contribute to levels of substances that are also neurotransmitters and c) have a role in neurophysiology have proved increasingly intriguing. In view of the complexity of the CNS, it is important to understand how MCs in the CNS might relate to those in the periphery and what role (if any) they might play within the brain. However, little is known about these cells in brain. Indeed, it was relatively recently that their existence in

the central nervous system (CNS) was considered controversial (Riley, 1959; Lagunoff, 1975). The studies presented in this thesis were designed to characterize brain MCs, quantify their numbers and distribution within rat brain and evaluate their HA content and contribution to brain HA and metabolite levels.

I) General characteristics and functions of MCs

MCs have a number of characteristics that are shared by other, non-MC cell systems. Therefore, the definition mentioned above must constantly be kept in mind when discussing these cells. Even within the MC classification, there is heterogeneity (see section I-G-i). With this in mind, the present use of the term MC will subsequently be used to denote only the connective tissue or serosal MC that has the following characteristics.

A) Morphological characteristics - MCs are round to oval, 10 to 25 micrometers (um) in diameter and contain a single mono-lobular, indented nucleus (see Selye, 1965). They have a poorly developed golgi apparatus and endoplasmic reticulum, and their mitochondria are neither large or numerous, suggesting that these cells are not normally involved in secretion (see Selye, 1965). The nucleus is usually positioned near the side of the cell and contains significant amounts of heterochromatin. The cytoplasm of MCs is filled with numerous moderate sized (0.1-1 um, mean approximately 0.6 um: Uvnas, 1978a) granules, to the extent that they

frequently obscure visualization of the nucleus. The stained granules are electron dense and are of homogeneous appearance (Uvnas, 1978a). These granules contain substances that give MCs a distinctive histochemical profile.

B) Histochemical characterization of MCs - visualization of MCs is dependent on the staining of components of MC granules. This can be accomplished with a wide variety of chemical dyes (Selye, 1965). Several methods have become widely used and are considered as standard in the classification of MCs.

i) Toluidine blue - toluidine blue is a cationic thiazine dye (Culling, 1974) which, when placed in aqueous solution, binds to charged anionic substances. Under appropriate conditions, this dye will change color from blue to reddish purple. This phenomenon, first described by Ehrlich (1879), is known as metachromasia. While toluidine blue is not the only dye capable of metachromasia (Schubert and Hamerman, 1956), its use for identifying MCs has become widespread, and the metachromatic staining of MCs has become part of its definition (see above).

The physical basis of metachromasia is not completely understood. Two schools of thought have arisen concerning this phenomenon, the polymerization or aggregation theory and the binding site theory. The binding theory holds that it is the binding of the dye to the anionic site that results in alteration of the electron configuration of the dye molecule, resulting in spectral shifts and metachromasia (Giles and

McKay, 1965). The aggregation theory, which is more widely accepted, holds that the binding of dye to anionic sites is a prerequisite, but that the binding serves to juxtapose dye molecules near each other and that this positioning results in dye-dye interactions of pi electrons. The electron configuration of the dye monomer is different from that of the polymer and hence metachromasia results (Michaelis, 1945; Culling, 1974). A widely accepted definition of metachromasia that does not depend on mechanism has been put forward by Padday (1970): "metachromasia is a characteristic reversible color change that any dye may undergo by virtue of a change in its environment not involving chemical reaction of the dye".

Several conditions are required for the appearance of metachromasia. Non-aqueous solvents, low temperature and high salt concentrations have deleterious effects on metachromasia (Schubert and Hamerman, 1956; Padday, 1970). The charge density is important, with only polymeric anionic substances being capable of eliciting metachromasia (Bank and Bundenberg de Jong, 1939; Schubert and Hamerman, 1956; Padawer, 1963). As only negatively charged substances can bind dye, the type of anion is important, with the stronger acidic groups retaining their charge at lower pH's than weaker acidic groups (sulfamates>sulfate>phosphate> carboxylate). The practical importance of this is the ability to increase the specificity of the dye by tailoring the pH of the dye solution to the nature of the groups being

stained. At higher pH's, all acidic polymers will be negatively charged and therefore be capable of exhibiting metachromasia. As the pH drops, polycarboxylate and then polyphosphate groups lose their charges until only the strongest of the organic acids remains, i.e., the sulfates and sulfamates (found in heparins, see Fig 3). Therefore, for the specificity of dye to be ensured, the pH must be closely controlled. PH's below 4 are required to prevent concurrent staining of other tissue components and to demonstrate the presence of highly sulfated glycosaminoglycan polymers. Under these conditions, this dye is highly specific for acid glycosaminoglycans such as heparin (Padawer, 1963).

ii) Astrablau FM - Astrablau is a copper phthalocyanin dye that has been shown to be valuable in the identification of acid glycosaminoglycans (Bloom and Kelly, 1960). Whereas toluidine blue is a metachromatic dye, Astrablau is orthochromatic, i.e., it does not undergo a color change once it has bound to an acidic substance. Thus, the use of appropriate pH conditions is even more important with this dye than it is with toluidine blue. Of interest is the observation that unlike toluidine blue, Astrablau staining is not labile in the presence of alchohol, making for a very stable stain (Bloom and Kelly, 1960).

iii) Ortho-phthalaldehyde (OPT) - this chemical, in the presence of small amounts of water, combines with certain amines to form fluorescent products. OPT is capable of

reacting with a number of substances, including histamine (HA), diamines, histidine and peptides with an NH_2-terminal histidine (Turner and Wightman, 1968; Brody et al., 1973). However, the spectral characteristics of these fluorophores differ (Ehinger and Thundberg, 1967), allowing the identification of the substances that fluoresce. Further, the conditions under which the fluorophore is formed can enhance the formation of specific fluorescent complexes, allowing the use of OPT for characterization of HA (see reviews: Hakanson, 1970; Lagunoff, 1975). This substance has therefore been used for the histological identification of HA in a number of cells, especially MCs.

The structure of the OPT-HA has not been equivocally identified. While the model put forward by Shore et al. (1959) has found wide acceptance, neither the intermediate nor final complexes had been isolated and identified. A recent study (Ronnberg et al., 1984) reports to have isolated and identified the final structure using HPLC and GC-MS, and identifies it as a dihydrophenantroline quarternary ammonium salt that is the product of 2 consecutive cyclization reactions with OPT (Fig 1).

iv) Paraformaldehyde - while the observation that formalin was capable of inducing fluorescence in mammalian tissues dates to 1938 (Feyrter, 1939), the breakthrough in the use of this method came with the development of the gaseous formaldehyde treatment of tissues for the fluorescent identification of 5-hydroxytryptamine (5HT) and catechol-

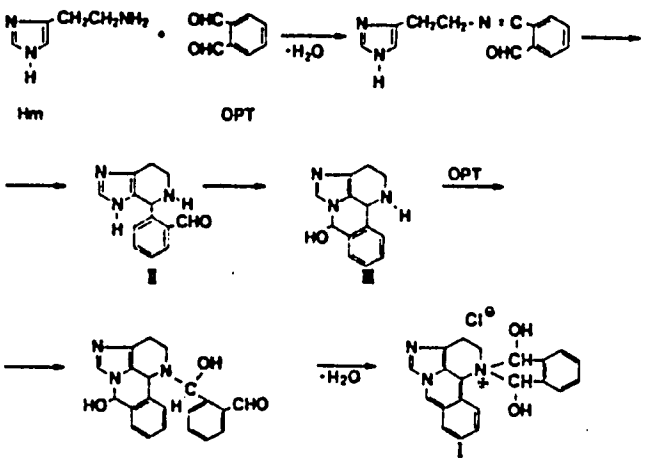

Fig 1. The proposed structure of the HA-OPT fluorescent adduct. HA combines with 2 molecules of OPT to form a quarternary ammonium salt (from Ronnberg et al., 1984).

amines (Falck and Torp, 1961). Since then, numerous modifications of this method have been developed, allowing for the identification of these compounds in a number of tissues and cells, including MCs (see review; Corrodi and Jonsson, 1969). In all cases, the final fluorescent product seems to be a cyclization of the condensation of formaldehyde with the primary amine to form tetrahydroisoquinolines and tetrahydro-beta-carbolines (Fig 2). Although formaldehyde might be capable of forming a complex with HA, it is not thought to do so under conditions used for the visualization for 5HT (Hakanson, 1970).

C) Species and tissue distribution of MCs

i) Species - MCs are found in almost all vertebrate species, including fish, amphibia, reptiles, birds and all mammals (see Selye, 1965). With the exception of some amphibians, the cells all share the morphological characteristics described above. Amphibian MCs, termed clasmatocytes (see Michels, 1963), are long and spindle-shaped, having long processes and containing "very fine and dust-like granules".

ii) Distribution within mammals - as indicated above, MCs are found in organs containing a significant connective tissue component. As such, they are found in a wide range of organs, including the liver capsule, heart, tongue, skin and G.I. tract. They have also been identified in the central nervous systems of a number of mammals (see Introduction section II).

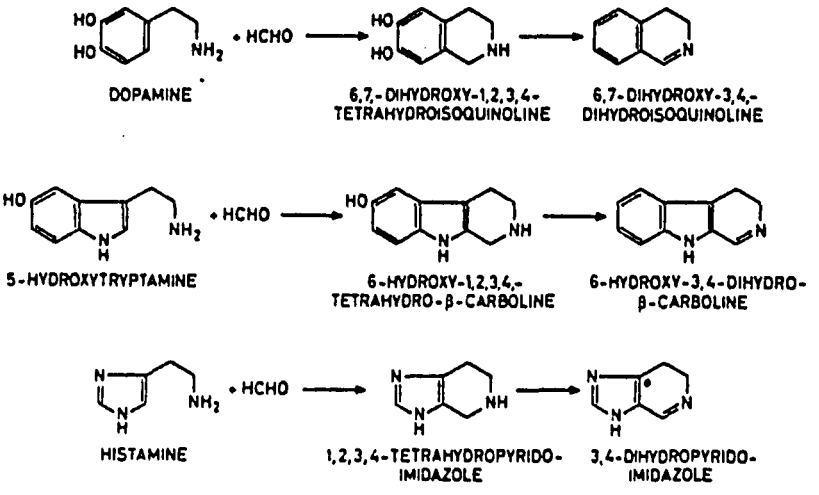

Fig 2. Proposed structures of adducts of dopamine, 5HT and HA following complexation with formaldehyde. Dopamine and 5HT adducts are highly fluorescent. HA does not seem to form such a product (from Hakanson, 1970).

D) Contents of MC granules (see the review by Wasserman, 1979)

i) HA and its metabolite tele-methylhistamine (tMH) - perhaps the most important substance found in the MC is HA, the beta-imidazole-ethylamine product of the decarboxylation of histidine (see Fig 9, Introduction section II). The content of HA in MCs varies considerably between species (Schmultzler et al., 1978) and between tissues of the same species (Erjavec, 1982). Values from 1 pg/cell (human lung MCs: Paterson et al., 1976) to 29 pg/cell (mouse peritoneal MCs: Barrett and Pierce, 1983) have been published. Rat peritoneal MCs contain 20 to 30 pg of HA per cell (Lagunoff, 1975; EnerbacK and Wingren, 1980; Kruger, 1980; Goldschmidt et al., 1984a). The turnover of HA in rat peritoneal MCs is very slow, with a half-life of approximately 23 days (Wingren et al., 1983a)

While the methylated product of HA metabolism (tMH, see Fig 9) has been shown to be present in rat peritoneal MCs (Goldschmidt et al., 1984a), the levels were extremely low (1/600 that of HA). This, plus the lack of histamine methyltransferase (HMT) activity in the cells (Furano and Green, 1964; Goldschmidt et al., 1984a) seems to indicate that there is little or no turnover of HA in these MCs. The levels of other HA metabolic products in MCs is unknown.

ii) 5HT - another amine found in MCs is 5HT. However, the levels of this mediator are usually much less (10 to 100 times) than that of HA (rat peritoneal MCs: Enerback and

Wingren, 1980). As with HA, 5HT turnover is slow, its half-life being on the order of 25 days (Wingren et al., 1983a).

iii) Enzymes - MC granules contain a number of different enzymes, including trypsin, chymotrypsin, chymase, arylsulfatase, N-acetyl-beta-D-glucosaminidase, beta-glucuronidase, beta-hexosaminidase, beta-galactosidase, peroxidase, superoxide dismutase, acid phosphatase, chloracetate esterase, histidine decarboxylase and 5-hydroxytryptophan decarboxylase (Wasserman, 1979; Lagunoff and Benditt, 1959; Lagunoff et al., 1962; Hall, 1966; Schwartz and Austin, 1980; Chakravarty, 1983).

iv) Peptide factors - MCs contain or generate a number of peptides that exert profound effects on the immune system once they are released. Included in this category are platelet activation factor (generated), eosinophil chemotactic factor (preformed) and neutrophil chemotactic factor (preformed) (Wasserman et al., 1974; Wasserman, 1979). An interesting observation is the presence and release of vasoactive intestinal polypeptide from rat and mouse MCs (Cutz et al., 1978).

v) Glycosaminoglycans - a large portion of the content of MC granules (approximately 25% of the organic content; Jaques et al., 1977) consists of highly sulfated polyanionic polymers called glycosaminoglycans (or sulfomucopoly-saccharides). These structures consist of polysaccharides of differing sulfated and carboxylated hexoses (Fig 3). The major portion of these substances consists of heparin (Green

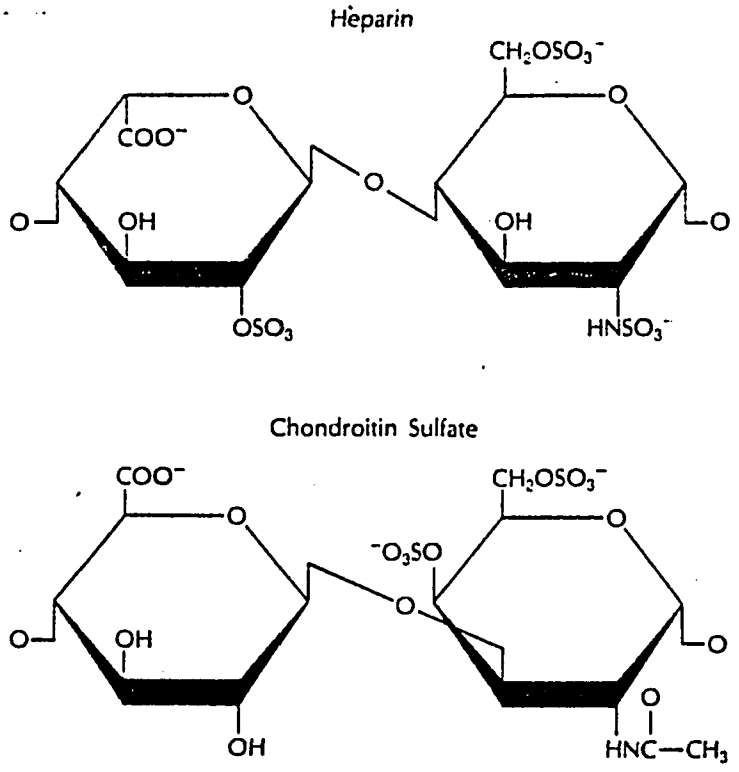

Fig 3. Structure of MC proteoglycans. These molecules consist of a protein core (not shown) to which is bonded side chains of highly sulfated repeating sugars (shown). Heparin contains iduronic acid and N-sulfated glucosamine. Chondroitin sulfate contains glucuronic acid and N-acetylgalactosamine-4-sulfate (from Austen, 1984).

and Day, 1960; Yurt et al., 1977). However, this name should be considered a generic one, as there is a heterogeneity in the structure of these substances in MCs of different tissues and species (Green et al., 1963; Jaques, 1982). Whatever the specific structure, it is the glycosaminoglycan of MC granules that confers the distinctive metachromatic capability to these cells in the presence of cationic dyes (see section I-B-i).

In MCs, it has been hypothesized that glycosaminoglycans do not reside in a free state, but are complexed through their sulfate groups to a highly basic protein that also resides in the granules (see review by Uvnas, 1978 a and b). The free carboxylate groups of the resulting proteoglycan can behave as weak cation exchangers and bind various cations (Fig 4). Indeed, it is to this "resin" that HA and 5HT (and many other biogenic monoamines under experimental conditions) are bound (Bergendorff and Uvnas, 1973).

The turnover of glycosaminoglycans in rat MCs, as measured by the elimination of ^{35}S that had been incorporated into MC granules, is very slow, with a half-life of about 35 days (Wingren et al., 1983a). This is not different from the turnover of HA and 5HT in these cells, and is further evidence that heparin or its cogeners are involved in the storage of amines.

Aside from its anticoagulant properties, heparin has been implicated in demyelinization and Wallerian degeneration processes through its ability to stimulate the

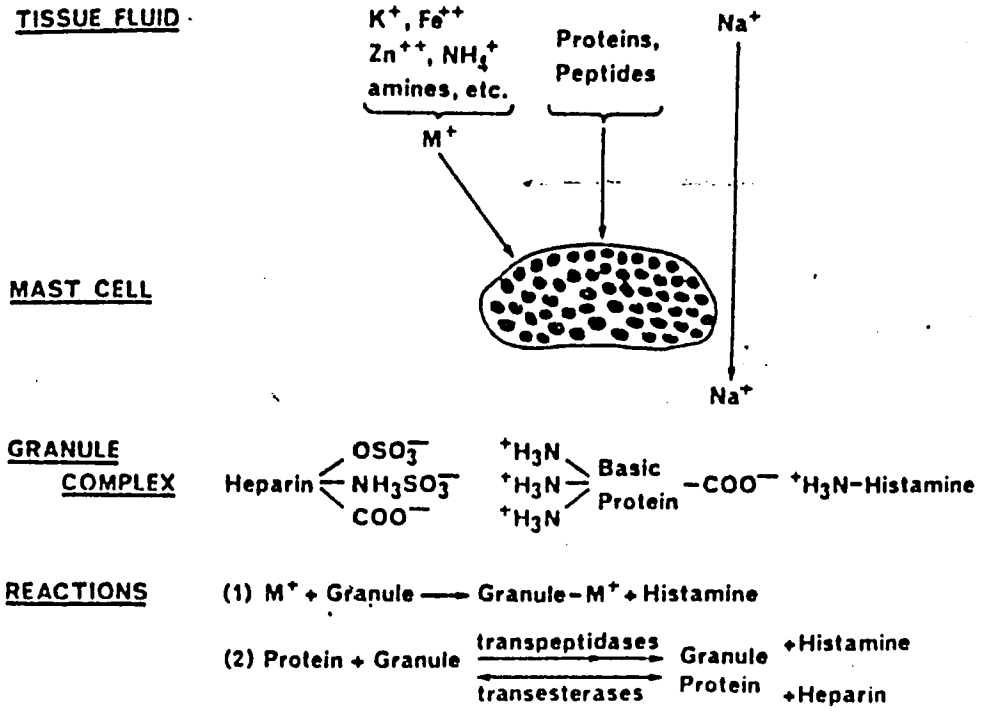

Fig. 2. Mast cells as ion exchangers. As indicated diagrammatically, cations (with the exception of sodium) enter the cell and exchange with histamine in the granule. Proteins are initially taken up by granules as cations and then are fixed to granule protein by transpeptidases by exchange with histamine or transesterases by exchange with heparin. Heparin is fixed to the amino groups of the granule protein through sulfate groups making available carboxyl groups for binding histamine without marked change in pH. (Modified from Ref. 17.)

Fig 4. MCs as ion exchangers. It has been hypothesized that heparin is bound to a protein core within the granule through its sulfate moieties. The free carboxyl groups of the protein can act as binding sites for cationic molecules, including amines, peptides and molecular cations (from Jaques, 1982).

release/activity of lipases (Enerback et al., 1965; c.f. Olsson, 1968) and in angiogenesis (Folkman et al., 1983).

vi) Arachidonic acid metabolites - while most of the substances discussed so far reside pre-formed in MC granules, at least 2 classes of mediators are formed and released from MCs at the time of stimulation and degranulation. These substances, products of arachidonic metabolism, are prostaglandins and leukotrienes (Fig 5).

a) Leukotrienes - these substances, previously called slow reacting substances of anaphylaxis (Samuelsson et al., 1980), are the products of arachidonic acid metabolism by the lipoxygenase system (see reviews by Borgeat, 1981; Hammarstrom, 1983). While there are several leukotrienes, leukotriene C is the primary compound released by MCs (Samuelsson et al., 1980). The substances have potent contractile activity on smooth muscle (Hedqvist et al., 1980), and therefore may be important in such anaphylactic symptoms as bronchiolar contraction and cardiac disfunction (see review: Piper, 1983). In addition, leukotriene B is a potent chemotactic agent for polymorphonuclear leukocytes (Ford-Hutchinson et al., 1980).

b) Prostaglandins - arachidonic acid can be metabolized by the cyclooxygenase pathway to yield prostaglandins and thromboxanes. Although a number of prostaglandins have been isolated, MCs preferentially generate prostaglandin D (PGD) (Austen, 1984). Although its potency in man is not known, this substance has been shown to have vasodilatory actions in

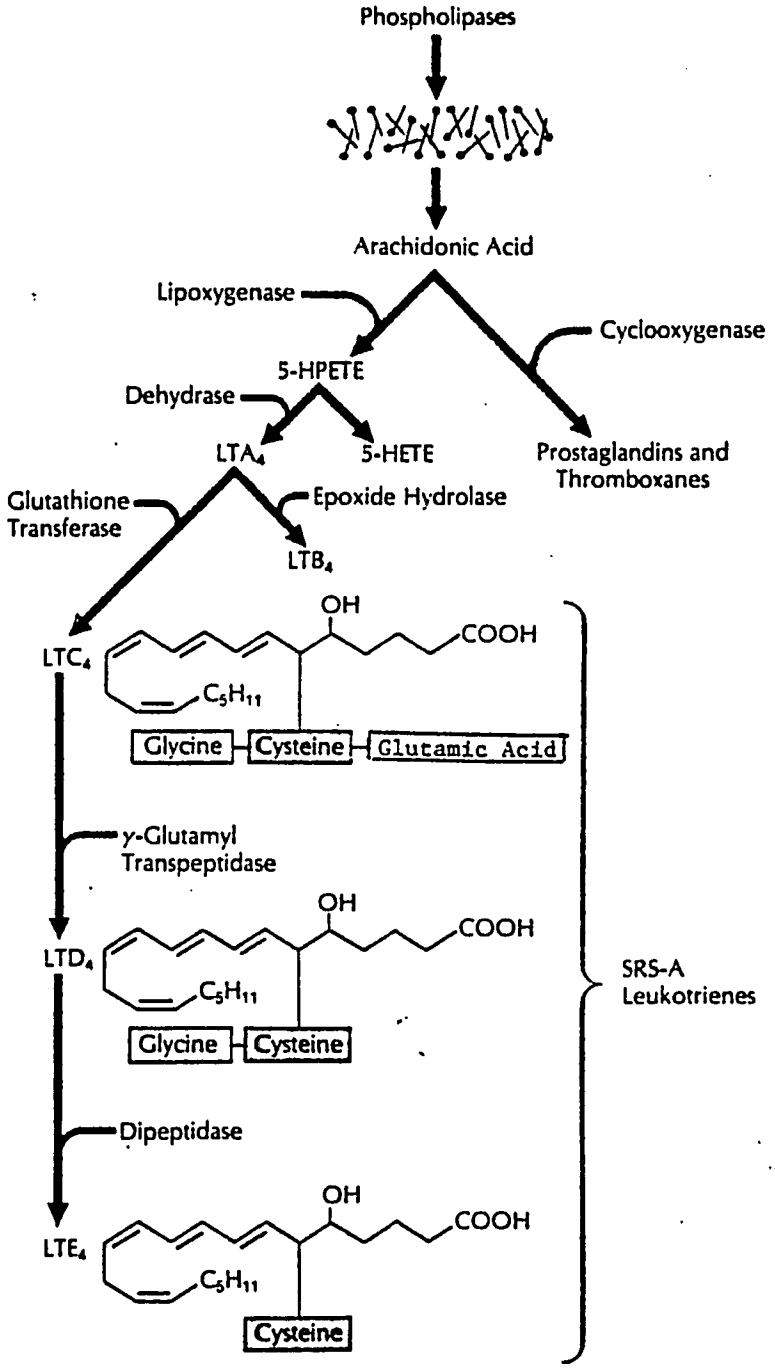

Fig 5. Metabolism of arachidonic acid in MCs to yield leukotrienes (LTs) and prostaglandins. LTC_4 is the primary leukotriene generated by MCs (from Austen, 1984).

several species (see review: Kennedy, 1982). Like other prostaglandins and similar to leukotrienes, PGD contracts smooth muscle, and can cause bronchoconstriction. This substance is a potent inhibitor of platelet aggregation in man, but has no such activity in rat or rabbit.

E) Uptake, storage and formation of HA and 5HT by MCs - rat MCs are capable of taking up biogenic amines from their surrounding medium. The uptake of HA has been shown to be a passive process, being concentration and time dependent while being independent of metabolic inhibition (Cabut and Haegermark, 1966; Green, 1966a and 1967). In contrast, the uptake of 5HT has been shown to be active, and MCs can take up considerably more 5HT than they can HA (Furano and Green, 1964; Jansson, 1970).

Although MCs can take up exogenous amines from their surroundings and incorporate them into the same areas as the endogenous amines (Cabut and Haegermark, 1966), it is clear that the majority of HA and 5HT residing in MCs is the product of the active uptake of the precursor amino acids and subsequent decarboxylation (Green, 1966 a and b; Lagunoff and Bauza, 1982).

F) Release of MC granules and contents - while it is possible that MCs might perform a physiological function due to spontaneous controlled release of the mediators they contain, the best characterized functions of these cells are due to direct external cell stimulation that results in immediate degranulation and subsequent dispersal of the

various substances contained within their granules. The sources of such stimulation fall into two categories, immunological and pharmacological.

i) Immunological stimulation of MCs

1) Immunoglobulin mediated non-cytolytic release - perhaps the most important syndrome in which MCs are active is the massive and immediate release of their mediators in the condition known as immediate hypersensitivity or anaphylaxis. An individual who has been exposed to a recognizably foreign substance (antigen) will develop a multi-level and sequential immunological response to that antigen. Among the many cellular responses will be the clonal replication of a number of B-lymphocytes whose function is to secrete various classes of immunoglobulins (Ig) that have binding sites specific for that antigen. Among these Ig types is IgE, a large (approximately 185,000 daltons; Bennich and Johansson, 1971; Fu-tong et al., 1980) globular protein whose one precisely known function is to interact with MCs.

MCs (and several other cell types including basophils and mucosal mast cells, see Introduction section I-H) contain in their surface membranes receptors specific for the Fc portion of IgE. These receptors consist of 2 subunits, an alpha chain of approximately 45,000 da that contains significant amounts of glycosyl residues and a non-carbohydrate containing beta chain of about 35,000 da (Metzger et al., 1983). The numbers of receptors for IgE (IgER) per MC is

highly variable, with a range of 1000 to 400,000 receptors per cell being found in one series of experiments (MacGlashan et al., 1983). The mean was determined to be approximately 85,000. The dissociation constant for solubilized IgER and IgE is high, being greater than 10^{-12} M (receptors isolated from rat basophilic leukemia cells; Metzger, 1977).

Following the first or primary exposure to an antigen and subsequent formation of the specific IgE, these molecules bind to the IgER on MCs. These MCs are now said to be "primed". If the antigen is polyvalent (i.e. has more than one area to which immunoglobulin can bind), a secondary exposure of the individual to this antigen will result in the binding of antigen to the IgE-IgER complex and the cross-linking of a number of the receptor complexes (Fig 6). This cross-linking sets into motion a number of discrete biochemical events that lead to the degranulation of MCs and release of the mediators held within these granules (see reviews by Sullivan and Parker, 1976; Kazimierczak and Diamant, 1978; Foreman, 1980; Ishizaka, 1981; Axelrod and Hirata, 1982; Patkar and Diamant, 1982; Siraganian, 1983; Grossman and Diamant, 1984).

Briefly, receptor-complex linkage leads to a simultaneous rise in intra-cellular cyclic AMP (cAMP) and to a series of membrane-bound enzymatic steps that convert phosphatidyl-serine to phosphatidylcholine (Fig 7). These phospholipid changes allow for the influx of calcium into the cell, the influx acting as the messenger for granule release (Ishizaka

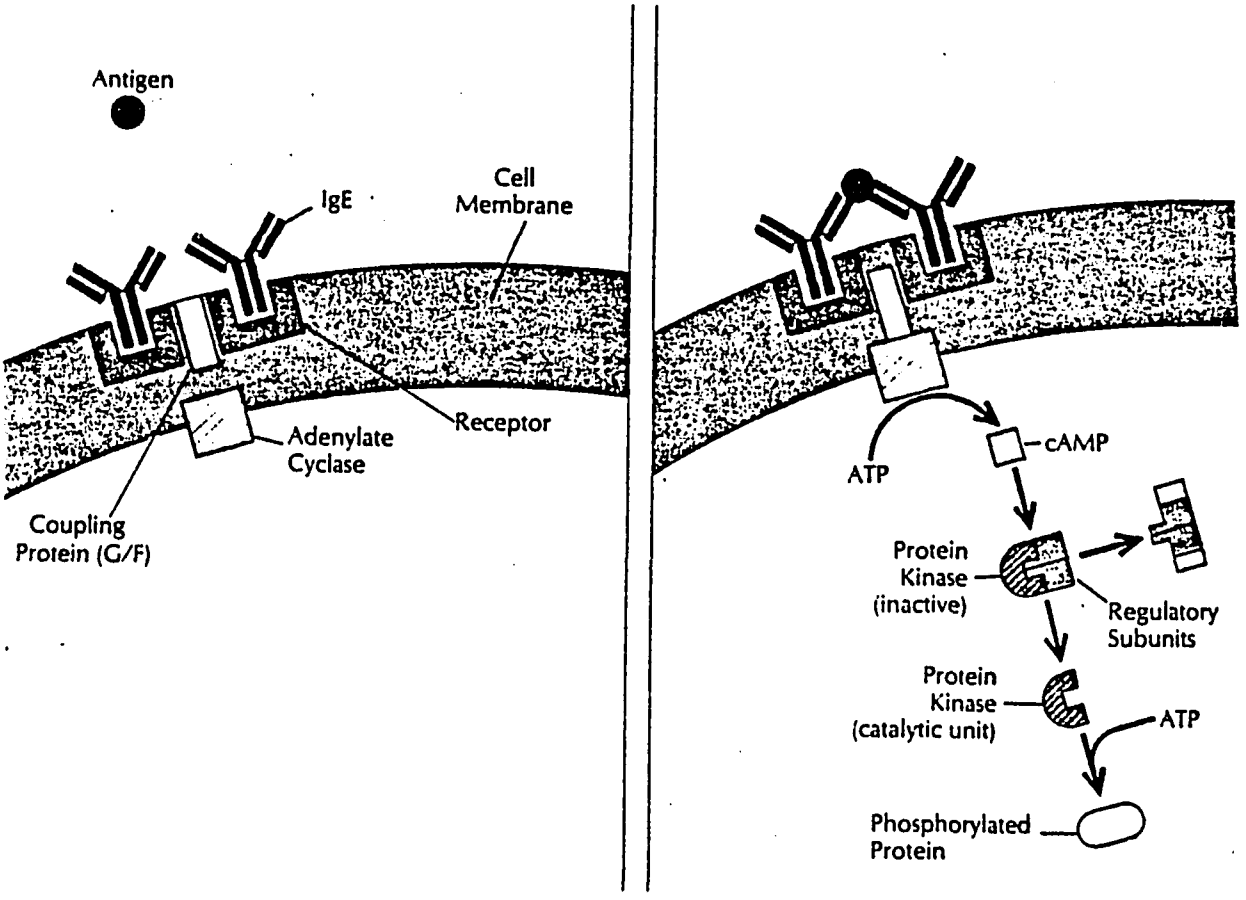

Fig 6. Cross-linking of IgE receptors on MCs resulting in degranulation. It is proposed that bridging of 2 receptors activates adenylate cyclase, which results in a cascade of kinase and methylation reactions (from Austen, 1984).

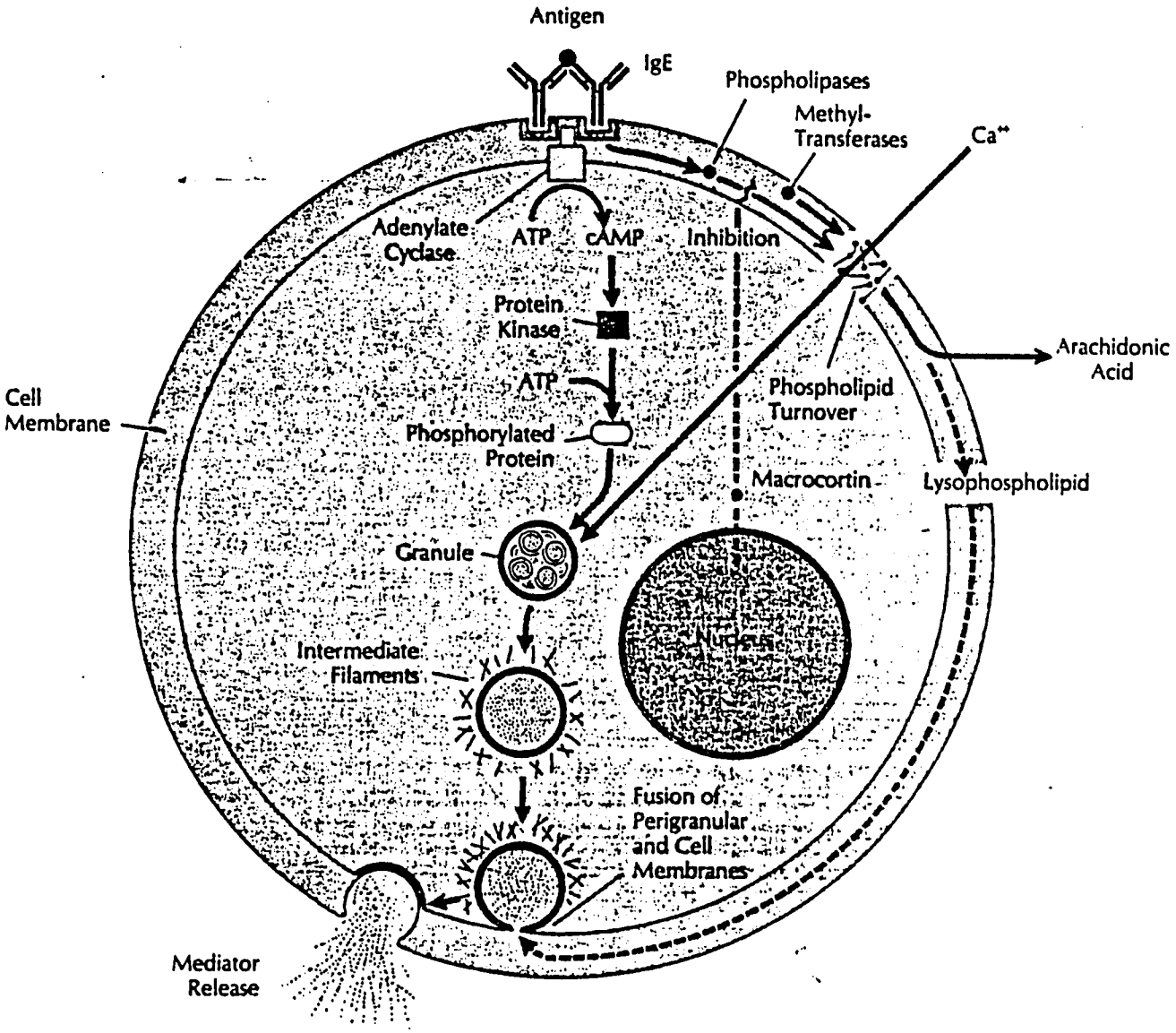

Fig 7. Biochemical events leading to MC degranulation following receptor linking (from Austen, 1984).

et al., 1981). There is evidence that microtubules are not responsible for the mechanisms of release (Lagunoff and Chi, 1978); rather, it is thought that the entry of calcium into the cell activates phospholipase A_2, resulting in the cleavage of phosphatidylcholine to form arachidonic acid and lysolecithin. The arachidonic acid goes on to form cyclo- and lipoxygenase products and the lysolecithin might act as a fusogen, promoting the extrusion of granules through the membrane (Siraganian, 1983). It must be noted, however, that while there is strong evidence for the involvement of calcium in message translation across the cell membrane, the 2 events (calcium influx and degranulation) may be simultaneous events that are not causally related (see review by Grossman and Diamant, 1984). This degranulation hypothesis holds that the rise in cAMP plays a negative modulatory role, and there is evidence that pharmacological elevation of cAMP (or cogener) levels in MCs results in the antagonism of release (Ishizaka et al., 1981).

In addition to IgER, rat MCs also have receptors for several sub-classes of IgG (Moodley and Mongar, 1981). However, the affinity of these receptors for IgG is much lower than that of the IgER for IgE, being on the order of 10^{-5} M (Siraganian, 1983). Although it might be different in other species, in the rat it is IgE and its receptor that is the primary pathway for immediate hypersensitivity.

2) Complement mediated non-cytolytic degranulation - among the many receptors found on MCs are those that bind

polypeptide fragments of the cleavage of several components of the complement system. The system is triggered by antigen-antibody complexes to initiate a cascade of enzymatic cleavages of plasma proteins, resulting in lysis of invading cells and release of chemotactic and degradative mediators from activated immune cells (Benacerraf and Unanue, 1979). Studies have shown that fragments C3a and C5a are capable of MC degranulation in a manner that is similar to that of IgE mediated release (see reviews by Goth and Johnson, 1975; Lagunoff et al., 1983).

ii) Pharmacological release of MC mediators

1) Compound 48/80 - a condensation product of formaldehyde and para-methoxyphenylethylmethylamine (Fig 8)(Baltzly et al., 1949), this compound was recognized to be capable of releasing endogenous HA by Paton (1951). The precise active form of this polymer is not known, as commercial preparations are a mixture of several lengths, from tetra- to octamers (Read and Lenney, 1972). However, trimers and tetramers are ineffective (DeGraw et al., 1966 a and b) and a preparation enriched in the hexamer was more potent than the commercial mixture (see Lagunoff et al., 1983). Although the precise mechanism by which 48/80 degranulates MCs in not known, it is believed that it interacts somewhere along the same biochemical path as that followed by IgE mediated release, and results in a fast but sub-maximal non-cytolytic degranulation (Johnson and Moran, 1969). Although IgE release is dependent on external calcium

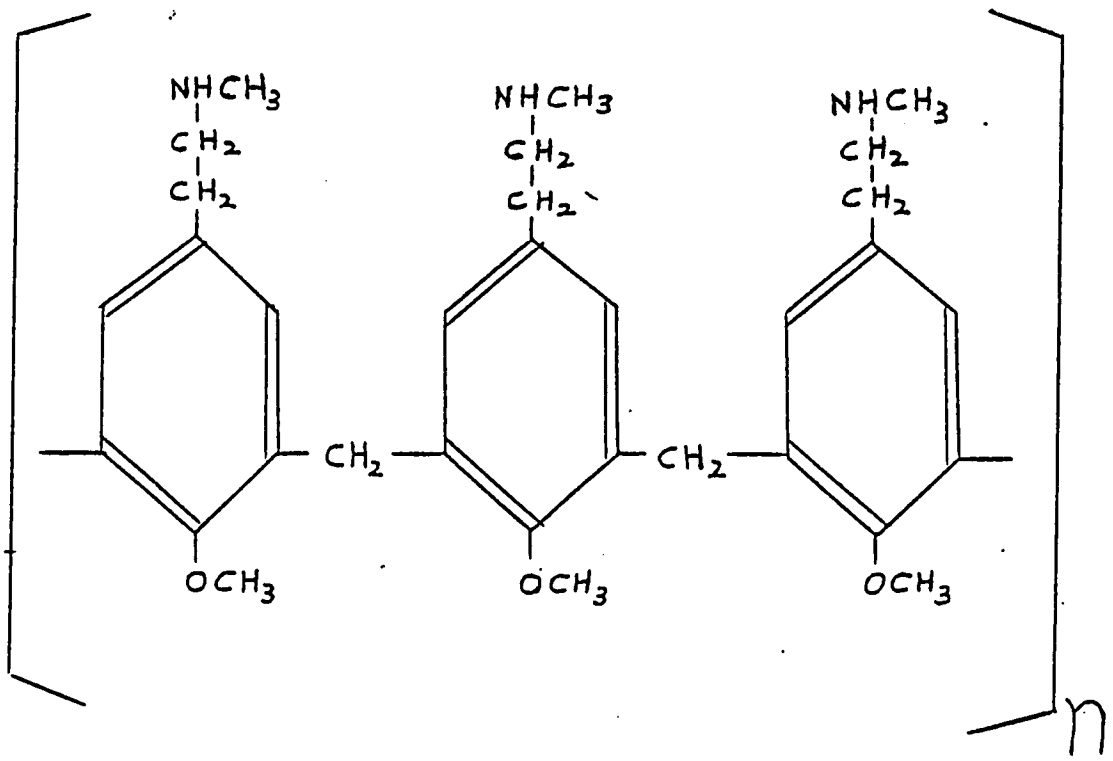

Fig 8. Structure of compound 48/80. The length of the active agent is not known (as Paton, 1951).

(either in surrounding medium or loosely bound to the outside of the plasma membrane; Pearce et al., 1981), release by 48/80 is not so restricted, and is able to utilize internal pools of calcium (Chakravarty and Yu, 1984).

2) Release by polybasic compounds - MCs are capable of degranulation by a number of polymeric basic substances, including a number of polypeptides and the antibiotics polymyxin B and E (Peachell and Pearce, 1984; review by Lagunoff et al., 1983). All these compounds seem to share similar mechanisms of release with that of compound 48/80.

3) Calcium ionophores - some of the evidence that calcium is an important signal for the initiation of degranulation is that the artificial elevation of internal calcium levels is sufficient to begin the process. This has been performed non-pharmacologically through the novel techniques of direct microinjection (Kanno et al., 1973) and fusion of MCs with liposomes filled with calcium (Theoharides and Douglas, 1978). Similar results have been obtained through the use of calcium ionophores (drugs that bind calcium and transport it across cell membranes). The most prevalent ionophore in present use is A23187, which has been shown to be active in a number of MC studies (Pearce and Truneh, 1981; reviews by Kazimierczak and Diamant, 1978; Lagunoff et al., 1983). Recently, the antibiotic chlortetracycline has also been shown to be an effective calcium ionophore and tool for MC degranulation (West, 1983; Pierce at al., 1983).

4) Morphine - this basic drug has been shown to be capable of selective non-cytolytic MC degranulation (Ellis et al., 1970; Grosman, 1981). However, the high concentrations required in some systems (greater than 20 mM) makes this activity of morphine of doubtful physiological significance. There is indirect evidence that this activity of morphine might be the due to a specific opioid receptor on the MC plasma membrane (Yamasaki et al., 1982). Morphine is an example of a number of drugs that have been shown to have non-cytolytic HA releasing capability. Others are clonidine (Lakdawala et al., 1980), codeine, tubocurarine, ketotifin and guanethidine (see review by Lagunoff et al., 1983). A number of drugs, including octylamine (Lakdawala et al., 1980), decylamine and chlorpromazine act in a cytolytic manner, lysing the MC membrane and causing granular extrusion through disruption (see Lagunoff et al., 1983).

5) Mitogens - certain plant lectins (or hemaglutinins) such as phytohemaglutinin (PHA), wheat germ agglutinin (WGA) and Concanavalin A are capable of significant and rapid MC degranulation (see reviews by Kazimierczak and Diamant, 1978; Lagunoff et al., 1983). The mechanism of action seems to be their ability to bind saccharide groups, in this case on the Fc region of receptor-bound IgE. As these substances are polyvalent toward saccharides, they act as cross-linking agents, in essence mimicking anti-IgE antibodies. It has not been ruled out that these substances might also act directly on surface structures.

6) Separate release of HA and 5HT from MCs - as the hypothesis put forward for the granular storage of MC mediators postulates that the binding sites for all amines are the same, it was of great interest that N-(alkyl)-3-hydroxybutyramide (butoctamide)(2.3 mM), in the presence of epinephrine (0.27 mM) was capable of degranulating rat MCs and causing the relatively specific release of 5HT but not that of HA (Ichikawa et al., 1977). Butoctamine alone showed no such activity. As the cells were degranulated, it seems likely that the release of HA was inhibited rather than the release of 5HT being promoted. An earlier observation (Miller and Church, 1976) that HA and 5HT release during pinnal anaphylaxis in mice was sequential rather than parallel lends some credence to this possibility. Subsequently, Theoharides et al. (1982) indicated that the tricyclic antidepressant amitriptyline (10^{-6} to 10^{-4} M) was capable of inhibiting the release of HA but not that of 5HT following 48/80 or antigen stimulation. Interestingly, this inhibition was accompanied by an inhibition of degranulation, prompting the authors to hypothesize that at least some MC 5HT might be stored outside of granules (in the cytoplasm) and that there might be a specific mechanism that allowed for the release of this substance. A recent report, that amitriptyline inhibits the release of 5HT but not HA in response to a number of pharmacological stimuli (Carraway et al., 1984), indicates that there might indeed be differences

in the binding and release of various granular bound constituents.

G) Pharmacological and histological heterogeneity of MCs - there are documented histochemical differences between MCs of different species (Spicer, 1963; Chiu and Lagunoff, 1972) and between MCs found within a single tissue type (Spicer, 1963; Leahu, 1972). While the MCs of all mammalian species contain HA, only rodent MCs contain 5HT (Benditt et al., 1963; Padawer, 1966) and only the MCs of ungulates contain dopamine (Coupland and Heath, 1961; Falck et al., 1964; Edvinsson et al., 1977). The morphology of MCs in different species has been shown to vary widely (Michels, 1963).

In addition to the histochemical differences between MCs, pharmacological investigations have shown a large heterogeneity of MCs between different species (Ennis and Pearce, 1980; Barrett and Pearce, 1983; Boarato et al., 1984; Leung and Pearce, 1984; Leung et al., 1984), between different strains of the same species (West, 1982; Brzezinska-Blaszczyk et al., 1984), between MCs from different tissues of an individual (Damas and Lecomte, 1983) and between MCs from a single tissue separated from each other by gradients (Wilhelm et al., 1978; Beaven et al., 1982; Schulman et al., 1983).

While it is beyond the scope of this thesis to discuss in depth all the complexities of the studies mentioned, there are several excellent reviews of the subject (Michels, 1963; Pearce, 1982; Erjavec, 1982; Pearce, 1983; Bienenstock et al., 1983; Barrett and Metcalfe, 1984). It is important to

define clearly the MC system used, and extrapolations from one model system to another may be limited.

H) Other cells of the MC family - as indicated at the beginning of this introduction, the definition of the MC is of necessity quite limiting, and leaves out several cells that may be functionally and/or morphologically similar to serosal MCs. The two most important members of this extended group are the mucosal mast cell (MMC) and the basophil.

i) the MMC - these cells, sometimes called atypical MCs, reside in the lamina propria of the intestine and are found only in the G.I. tract (Saavedra-Delgado et al., 1984). Like typical MCs, they contain basophilic granules, IgER, and contain many of the same mediators, including HA and 5HT (see reviews: Miller, 1980; Bienenstock et al., 1983; Guy-Grand et al., 1984). While MCs contain heparin, MMCs contain a glycosaminoglycan that is less sulfated, probably chondroitin sulfate E. They proliferate in response to intestinal parasites such as N. brasiliensis and there is evidence that a localized immediate hypersensitivity reaction may be instrumental in the expulsion of these organisms (Wingren et al., 1983b). These cells have been differentiated from MCs on the basis of their histochemical properties (staining sensitivity of aldehyde fixation and inability to stain with toluidine blue at low pH)(Enerback, 1966). Until recently, it was thought that this sensitivity to aldehydes was due to solubilization of the granules, but a masking of the dye binding sites by these fixatives has been implicated (Wingren

and Enerback, 1983). Unlike typical MCs, MMCs are refractory to the releasing activities of many of the polybasic agents such as 48/80 and bee venom 401 (Befus et al., 1982). These cells were also refractory to the anti-allergic drugs disodium cromoglycate, AH 9679 and theophyline (Pearce et al., 1982). Both types of MCs respond to calcium ionophores although MMCs did so to a lesser degree than did MCs (Pearce et al., 1982). An interesting observation is that while typical MCs contain IgE only on their surface, MMCs contained cytoplasmic as well as surface-bound IgE (Mayrhoffer, 1977). Whereas serosal MCs are not dependent on the thymus for proliferation and differentiation, MMCs are thymus dependent and are not found in athymic mice (Ruitenberg and Elgersma, 1976). It is likely that MMCs are similar or the same as the recently described cloned MCs (see below, section H) and therefore are dependent on soluble T-cell derived factors for proliferation and differentiation.

It is possible that a third described variant of the MC, called the globular leukocyte (see reviews: Gregory, 1979; Miller, 1980), is similar to the MMC.

ii) Basophils - while MCs have a non-lobulated nucleus, basophils belong to the myeloid family of leukocytes and are therefore multilobed (see reviews: Selye, 1965; MacGlashan et al., 1983; Lichtenstein et al., 1983; Dvorak et al., 1983; Metcalfe, 1983). Both cells contain metachromatic granules (although basophil granules contain chondroitin sulfate, while MC granules contain heparin, Orenstein et al., 1981)

and qualitatively similar mediators. Basophils tend to be smaller than MCs and have larger granules. While MC granules of most species tend to be homogenously electron dense (in humans they have an internal structure of whorls and scrolls), human basophil granules have a fine granular or crystaline structure (Katz, 1978). MCs contain higher HA levels than do basophils and basophils do not contain platlet activating factor (PAF). Whereas MCs are long-lived ($t_{1/2}$=months), basophils turn over quickly (circulation $t_{1/2}$=5.7 hrs; Parwaresch, 1976). While MCs seem to be incapable of movement, basophils can display ameboid motility (Selye, 1965). Finally, there seems to be a reciprocal relationship between MCs and basophils, with some species having high titers of circulating basophils and having low levels of tissue MCs, while other species have few basophils but many MCs (Riley,1959; Selye, 1965; Parwaresch, 1976).

I) Cloning and culturing of MCs - as seen through the information presented so far, the use of MCs as a model system for secretory events is widespread. Partly, this is due to the great range of the mediators that MCs contain and the profound actions of these compounds. Of equal significance is the fact that certain types of MCs are easily obtainable, by peritoneal lavage or more recently, enzymatic digestion of collagen in tissues that contain MCs, freeing them to be separated and harvested from other cell types. The drawbacks of these methods is that these cells are not viable over long periods once they have been purified, and

they do not divide in vitro. Further, the effects of isolation (collagenase and gradient purification) are not easily evaluated. A number of authors have opted instead to study mastocytoma cells, as these cells are capable of division and thus can be studied over long periods. However, mastocytomas are obviously not normal MCs and thus their use as model systems is open to question. Recent developments in the immunology of cell differentiation hold promise in alleviating some of these problems.

In 1981, three almost simultaneous reports were published concerning the ability of soluble factors derived from stimulated spleen cells or T-cells to stimulate the proliferation and differentiation of MCs from cultured bone marrow tissue (Nabel et al., 1981; Nagao et al., 1981; Razin et al., 1981). These cells were called P-cells due to their unusually long persistence in cultures (Schrader et al., 1981). While the vast majority of the research performed was done on murine tissues, similar cells have been cultured from human bone marrow (Horton and O'Brian, 1983). Subsequent investigation revealed that at least one of the factors involved is interleukin 3 (IL 3)(Razin et al., 1984), a glycoprotein produced by stimulated T-cells that has differentiation activities on mixed spleen cultures (Lee et al., 1982; Ihle et al., 1982).

While P-type cells have been cultured from several tissue sources, these cells all share similar characteristics and may be the same cells or of the same lineage at slightly

different stages of differentiation. P-cells contain HA, though at lower levels than do peritoneal MCs (Razin et al., 1981; Schrader et al., 1981). Similarly to MCs, these cells contain a chloroacetate esterase enzyme (Nagao et al., 1981). P-cells express similar numbers of IgER on their surface as do MCs and can be immunologically induced to degranulate and release their mediators (Razin et al., 1981). Similarly to MCs, P-cells form and release leukotriene C_4 upon stimulation, although they release much greater amounts of this substance than do MCs (Razin et al., 1982). Unlike MCs but similar to MMCs, P-cells contain chondroitin sulfate E and do not synthesize heparin (Sredni et al., 1983; Razin et al., 1984). Finally, although P-cells cloned from several different sources share a number of distinct surface antigens (as determined by monoclonal antibodies), these surface determinants are different from those found on serosal MCs (Katz et al., 1983). Interestingly, MCs not only bear surface antigens unique to themselves (Katz et al., 1983), but they also bear a determinant that had until recently been thought to be specific to macrophage lineages (Katz et al., 1981). The evidence has led investigators to classify these cells as MMCs or cells of a similar lineage, rather than being related to serosal MCs (Sredni et al., 1983; Guy-Grand et al., 1984).

I) Functions of MCs - aside from the role that MCs play in immediate hypersensitivity described above, there are several other proposed functions for MCs.

i) MCs may play a physiological role in situations other than degranulation in pathological syndromes according to Padawer (1979). Various substances are ingested or taken up by MCs, transported inside vesicles to various areas within the cell, acted on by various enzymes or bound to different compounds and then ejected out into the extracellular space. These modified antigens might then intercalate into MC membranes or bind to other areas and act as a long term source of antigenic memory, maintaining the immune system's recognition capability. According to this hypothesis, massive degranulation is not only an abnormal function, but is detrimental to the normal activity of the cell.

ii) MCs as a source of enzymatic initiation of biological activity - under this unusual proposal, the many enzymes contained within MC granules might act on the numerous substances that require cleavage to be converted from inactive to active forms (Theoharides et al., 1981). Some of the systems that use such a mechanism are the complement system factors, the clotting factors resulting in fibrin formation and the renin-angiotensin system involved in vascular homeostasis.

iii) MCs as an integral part of delayed hypersensitivity - delayed type hypersensitivity (DTH) is an immune response to foreign organisms or toxins that is characterized by a delayed (approximately 6 hrs post-introduction) inflammation and progressively severe indurated lesion (Benacerraf and Inanue, 1979). Unlike immediate hypersensitivity, in which

MCs release mediators into the blood stream, DTH is characterized by a massive local influx of phagocytically active leukocytes and macrophages which ingest foreign particles and disseminate various hydrolytic enzymes and factors. In the dermis, contact sensitivity is an example of DTH. In neuronal systems or the CNS, such syndromes become known as neuropathies and can involve such additional symptoms as demyelination and both local and systemic neuronal lesions (Arnason, 1975). Multiple sclerosis and encephalomyelitis are two of the many neurological syndromes that are in large measure DTH pathologies.

Recent evidence has suggested that while the primary recognition of antigens in DTH reactions is by T-cells, the MC may be a link whereby activated leukocytes and macrophages gain entry to sites of invasion (Gershon et al., 1976). These T-cells are said to release a specific factor(s) capable of MC degranulation, and the released mediators summon the influx of inflammatory cells across vascular linings and into surrounding tissue (Ashkenase et al., 1980; Van Loveren et al., 1983; Kops et al., 1984). Interestingly, these authors find evidence for an immunologically induced-difference in the release of HA and 5HT from murine MCs (Van Loveren et al., 1984).

Should the tissue MC be an integral part of DTH reactions, it would be expected that the mutant mast cell-deficient mouse (W/W^v; see Introduction section III-C-v) would show decreased DTH responses relative to +/+ control

animals. This is not the case, as W/W^v mice exhibit full responses (in some cases greater than controls)(Thomas and Schrader, 1983; Galli and Dvorak, 1984; Galli and Hammel, 1984). Thus, while MCs may play a role in DTH, their role is not exclusive, and redundant mechanisms may exist.

In summary, MCs show a wide distribution in both species and tissues. The cells are storage depots for a number of highly potent and diverse mediators, and these substances have activities in a number of pathological syndromes. In addition, there are several hypotheses linking MC function to normal physiology and homeostatic mechanisms. Thus, MCs are of widespread importance throughout peripheral systems.

II) MCs in the central nervous system (CNS)

Unlike MCs of the periphery, very little is known about these cells in brain. Until recently, their existence in brain was disputed (Riley, 1959; Lagunoff, 1975; El Akad and Brody, 1975) and most of the studies suggesting their presence were only concerned with histochemical identification. There is little data available relating to the contents of these cells and less is known pertaining to any functions these cells might perform within the CNS.

A) Presence of MCs in brain - the presence of MCs in brain has been described since the late nineteenth century (human brain in pathological conditions; see Selye, 1965). However, although several studies examining the mammalian CNS for MCs appeared through the 1920's, there was little

agreement as to the existence of brain MCs and even less concerning which species (if any) might consistently contain them (see Selye, 1965). As a result, no consensus developed as to the presence or absence of these cells in brain. Following the initial burst of interest, few papers were published on the subject from the 1930's to the early 1960's (see Selye, 1965). In 1964, beginning with a report that indicated the presence of MCs in the brains of hamsters (Kelsall and Lewis, 1964), several studies appeared which suggested the presence of these cells in the CNS of both hamsters (Kelsall, 1966) and hedgehog (Campbell and Kiernan, 1966). Subsequently, analyses of the differential species distribution of brain MCs suggested that the cells were also present in the brains of rodents (Dropp, 1972,1976; Ibrahim, 1974; Kruger, 1974; Edvinsson, 1977; Persinger, 1977-1983), hedgehogs (Campbell and Kiernan, 1966; Kruger, 1970; Flood and Kruger, 1970), hamsters (Kelsall, 1966), carnivores, elephants (Dropp, 1976) and cats (Cammermeyer, 1973; Edvinsson, 1977). MCs have been reported to be absent from the brains of rabbits, guinea pigs, dogs, primates and human (Ibrahim, 1974; Kelsall, 1976), but other investigators claim to have found cells in the brains of these species (Cammermeyer, 1972,1973; Edvinsson, 1977; Dropp, 1976,1979).

A number of histochemical stains have been used by the investigators listed above. Several reports have used toluidine blue (rodents and primates-Cammermeyer, 1972; rats-Kruger, 1974; Ibrahim, 1974; Dropp, 1976; several species

including rat- Edvinsson et al., 1977), while others have used cresyl violet (rodents and primates- Cammermeyer, 1972; rats- Ibrahim, 1974; Dropp, 1976) or Astrablau (rats-Kruger, 1974; Edvinsson et al., 1977). However, the conditions under which these stains have been used have varied and this has led to some confusion as to the absolute identification of these cells.

As discussed (Introduction section I-B-i), the pH of the dye solutions used for the identification of MC glycosaminoglycan is critical to its specificity. Below pH 3.5, only MC heparin will stain metachromatically with toluidine blue, and other non-metachromatic stains (Astrablau) exhibit increased specificity. For this reason, it is imperative that the conditions under which "MCs" are identified be clearly described (Padawer, 1963). Although several studies have indicated that the staining protocols used for the visualization of brain MCs with toluidine blue and cresyl violet were appropriate (pH less than 4: Cammermeyer, 1972,1973-primate, cat, dog; Ibrahim, 1974-numerous species including rat, mouse,hamster, guinea pig and primate; Kruger, 1970-hedghog,1974-rat), others have been less specific in their descriptions, not mentioning anything except the type of dye used (Dropp, 1972,1976-rat; Edvinsson et al., 1977-several species including mouse, rat, rabbit, hamster,guinea pig, primate, human; Campbell and Kiernan, 1966-hedghog; Kiernan, 1976-numerous species including rat, mouse, hamster, hedghog, guinea pig, dog, cat, primate,

human; Kelsall, 1966-hamster). Kelsall and Lewis (1964-hamster) used toluidine blue at a marginally acidic pH (4.7) while another group (Mares et al., 1979) identified MCs in rat brain using neutral toluidine blue (pH 6.7) and was unable to identify them at a lower pH.

Further confusion has resulted from the use of Astrablau for the identification of brain MCs. All authors who have used this dye indicated that they used the protocol set forth by Bloom and Kelly (1960). However, this paper provides three methods of staining ranging from 2% Astrablau acidified with HCl (pH 0.2) to 1% Astrablau acidified with acetic acid (pH 4.1). Thus, in none of these papers is the precise method that was used clearly delineated.

Aside from the diversity of conditions used for the identification of brain MCs, no studies have reported that the varying stains used were visualizing the same population of cells. Kruger (1974) indicated that similar numbers of rat brain MCs were obtained when using toluidine blue and Astrablau, but did not counterstain sections, thus preventing direct comparison. Although Edvinsson et al. (1977) indicated that brain MCs from several species (including rat, mouse, rabbit, hamster, primate, human) stained with Astrablau and toluidine blue, no attempt was made to compare the two stains. Thus, no evidence has been published to date that conclusively shows that brain MCs bind more than one stain under appropriate conditions and that these cells are identical.

Because of the lack of a unifying analysis of brain MCs with several histochemical stains at appropriate pH's and the lack of evidence that a single population of cells is capable of binding all these dyes under differing conditions, the first goal of this thesis was to use both toluidine blue and Astrablau at acidic pH to identify unequivocally rat brain MCs and to determine if the same population of cells binds both dyes, just as MCs found in peripheral tissues are known to do.

B) Regional distribution of brain MCs - brain MCs do not seem to be distributed randomly or ubiquitously within different brain regions. MCs have been found to be congregated in thalamic areas of the rat (Ibrahim, 1974; Dropp, 1972,1976; Persinger, 1977,1979,1980; Kruger, 1974), hedgehog (Kruger, 1970; Kiernan, 1976), hamster (Kelsall, 1966) and of a number of other species, including primates, felines and hedgehog (Cammermeyer, 1973; Dropp, 1976; Kiernan, 1976). Other areas reported to contain MCs are choroid plexus (hamsters: Kelsall and Lewis, 1964; gerbil: Dropp, 1972; human: Dropp, 1979; monkey: Dropp, 1976) and area postrema (several species: Cammermeyer, 1972; Dropp, 1979). While MCs have been observed in cortical areas, they do not usually appear to make up a large portion of the total brain MC number (especially in rodents: Dropp, 1972,1976; Kruger, 1974; Persinger, 1979). With the exception of one report (Edvinsson et al., 1977), MCs have not been identified in rat basal hypothalamic nuclei with histochemical tech-

niques. A few MCs were reported to be present in hypothalamic nuclei in mice (Ishibashi and Sawano, 1982).

While various histological reports relating to brain MCs have indicated that these cells have a distinct brain distribution centered within the thalamus, almost none of these studies were quantitative and none were able to provide firm estimates of the precise contribution of any brain region to the total MC number in brain. Dropp attempted such an analysis in albino rats, but was hampered by using few animals (1972,n=3; 1976,n=5 of each sex) and an incomplete regional examination (1972: several regions including thalamus; 1976: only cotrex and dorsal thalamus). As a number of studies examining the biochemical parameters of rat brain HA have established the presence of significant non-neuronal pools of HA in non-thalamic areas and have attributed this HA to MCs (Introduction section III), it is important to establish firmly the regional distribution of these cells before attempting to attribute any function or HA contribution to them. Therefore, the second major goal of this thesis was to characterize in detail the regional localization and quantitative distribution of rat brain MCs.

C) Quantification of MCs

i) Thalamic distribution of rat brain MCs - relatively little work has been done to quantify the MCs of mammalian brain. MCs were reported to be found in the lateral, lateral geniculate and ventral nuclei of the thalamus of adult kangaroo (n=11) and albino rats (n=3)(Dropp, 1972).

Major concentrations were subsequently found in the medial geniculate, lateral, medial, paraventricular, posterior, ventral, lateral geniculate nuclei and the stria medullaris of several rodents (no quantitative data provided)(Dropp, 1976). Concentrations of MCs within specific thalamic nuclei have been reported in 21 day old rats (ventral, anterior ventral, reticular and parafascicular nuclei; Persinger, 1979), and hedgehog (medial dorsal nucleus-n=10; Campbell and Kiernan, 1966: habenular, medial dorsal, lateral and geniculate nuclei-n=27; Kruger, 1970). Persinger (1977a) mentioned observing a similar thalamic nuclear distribution in rats as that reported by Dropp (1976), but provided no data.

While the studies outlined above examined the thalamic nuclear distribution of brain MCs, none of the analyses (with the exception of Dropp, 1972, who studies 3 albino and 11 kangaroo rats) were quantitative. All studies indicated which nuclei contained concentrations of MCs, but no information as to the precise contribution these nuclei made to the total thalamic MC numbers was presented. Data concerning the actual numbers of MCs per nucleus was similarly lacking. Dropp (1972) provided quantitative data, but used few animals (see above). Further, total thalamic MC numbers seemed low relative to those found by others (see below).

Ages of the animals used in these studies have been inconsistent. Dropp used rats that were 1 to 1.5 years old

(Dropp, 1972) or whose ages were unknown (Dropp, 1976). Similarly, Persinger used rats that were 3 weeks old (1977b) or were of indeterminate age (1977a).

Similar to the nuclear distribution of brain MCs, little is known about the A-P distribution of these cells. A "standing wave" of MCs in rat thalamus was mentioned in a review, but no data were shown (Persinger, 1977a) and a bimodal curve of these cells was observed in this region in 21 day old rats (Persinger, 1977b). However, the density of MCs in thalamus is not known. Therefore, estimates cannot be made as to whether these cells are evenly distributed as a function of volume, or whether there is a heterogeneous density distribution.

ii) Variability in thalamic MC numbers - although quantitative studies of the MCs of thalamus have been few, it has become very clear that the number of brain MCs can vary a great deal between individuals of the same species. Dropp (1976) found a range of 333 to 4358 MCs and of 1509 to 3454 MCs in the brains of 5 male and 5 female adult albino rats, respectively. This observation confirmed an earlier report which found 327 to 1205 MCs in the brains of 3 albino rats and of 36 to 1951 MCs in the brains of 11 kangaroo rats (Dropp, 1972). Similar results were found in other species (albino mice-36 to 3824 MCs, n=5; meadow vole-28 to 2546, n=4; squirrel monkey-0 to 1416 MCs, n=4; Dropp, 1976). Persinger found that 3000 to 45,000 MCs resided in the brains of 21 day old albino rats (1979) and 5230 to 10144 MCs in the

thalamus of twelve 100 day old rats. For this reason, not only is quantification of brain MCs difficult, but reports of mean values based on small numbers of animals must be considered only partially representative of the true population.

In view of the lack of studies available on the numbers and distribution of thalamic MCs, the MCs of rat thalamus were quantitatively evaluated as a function of both coordinate and nucleus, and the relationship of these factors to total thalamic MC numbers was explored.

D) Ontogenic changes in rat brain MC numbers and regional distribution

Rat brain MCs undergo striking changes in both numbers and distribution as the animal matures. MC numbers are high in the neonate and they are situated in the meninges over cortical areas (Dropp, 1976; Ferrer et al., 1979; Persinger, 1981). As the animal matures, these numbers drop and the distribution changes from the meninges to the thalamus. Ferrer (1979) and Persinger (1981) observed that these shifts were substantially completed by day 21, with total MC numbers being stabilized at levels similar to those in adults and with greater than 85% of total MCs residing within thalamus. At this stage, few MCs were found in meninges. Dropp (1976) observed similar changes, but found that these trends were not completed until after the animal was older than 6 months. It is not known whether the ontogenic change in brain MC distribution is due to movement of meningeal MCs to thalamus,

or the growth and differentiation of new thalamic MCs in situ.

III) Chemical and histochemical characterization of brain HA: relationship to neurons and MCs

HA has been of pharmacological interest since its description in pituitary and other tissues (Abel and Kubota, 1919). Since then, HA has been found to be present in numerous tissues and to be physiologically active in several peripheral organ systems, including the gastrointestinal (see reviews: Bunce et al., 1979; Beaven, 1982) and the immunological systems (see review: Plaut and Lichtenstein, 1982). Interest in HA has increased due to evidence that it exists in brain and might play a role in neuronal transmission.

Attempts to characterize brain HA and its precise cellular localization have taken several routes: regional level comparison, ontogeny, lesions of brain tissue, turnover studies, histo- and immunohistochemistry, inhibition of various pathways in the HA metabolic pathway and the use of mutant mice deficient in MCs. These studies have provided evidence that brain HA resides in both neuronal and non-neuronal pools.

A) Formation and metabolism of HA

i) Structure and formation of HA - this substance is the imidazole ethylamine product of the decarboxylation of histidine (Fig 9). Although there are two enzymes capable of

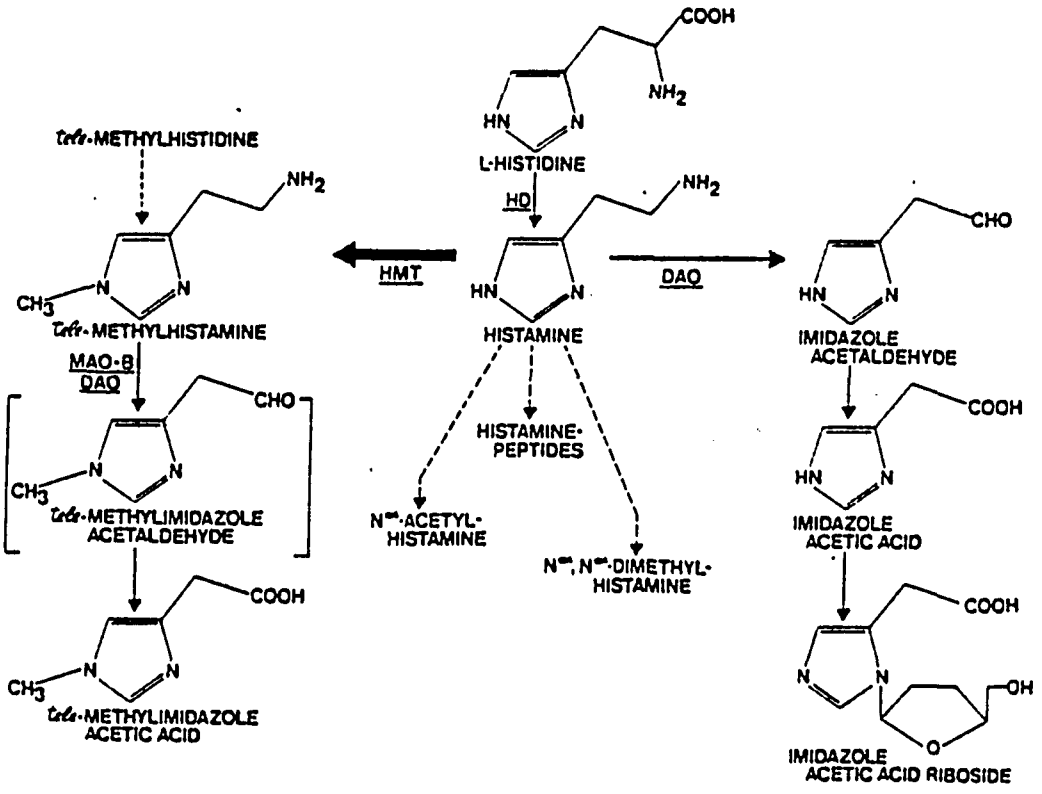

Fig 9. Metabolism of HA. HA is formed by the decarboxylation of histidine by histidine decarboxylase (HD). HA can be oxidized by diamine oxidase (DAO) to imidazole acetic acid or be methylated by histamine n-methyltransferase (HMT) to tMH. As mammalian brain lacks DAO, methylation is the only pathway of HA catabolism. Brackets indicate presumed aldehyde intermediate that has not been isolated. Dotted lines indicate possible minor pathways in brain (from Hough and Green, 1984).

the formation of HA, only the specific enzyme histidine decarboxylase (HD, EC 4.1.1.22) is responsible for the synthesis of HA in brain (see Hough and Green, 1984). As HA is charged at physiological pH, it does not penetrate the blood brain barrier easily and must be formed in situ within the brain (Schayer and Reilly, 1970; Bulfield and Kacser, 1975).

ii) Metabolism of HA in brain tissue - while oxidation of HA to imidazolacetic acid by diamine oxidase does take place in the periphery, methylation of HA to tele-methylhistamine (tMH) by histamine n-methyltransferase (HMT, EC 2.1.1.8) is the primary if not exclusive route of HA degradation in rat brain (see reviews: Green, 1970; Bunce, 1979; Beaven, 1982; Hough and Green, 1984).

B) Regional distribution of HA in rat brain

i) Histochemical visualization of rat brain HA - the results of attempts to visualize brain HA with the gaseous OPT method (see Introduction section I-B-iii) have been mixed. There are no reports of the successful visualization of neuronal HA with this technique and several authors have been unsuccessful in visualizing HA in brain MCs (Hakansson, 1970; Ibrahim, 1975; El-Akad and Brody, 1975). The reason for the inability to visualize HA in brain MCs has been a described autofluorescence of the granules. Edvinsson et al. (1977) were successful in using OPT to show that meningeal MCs contain HA. As the identification of HA in brain MCs is essential to the characterization of these cells and to the

hypothesis that brain MCs contribute to brain HA levels, a goal of this thesis was to use the gaseous OPT method to determine if brain MCs exhibit HA fluorescence and to see if the fluorescent cells were the same cells that stain metachromatically with toluidine blue.

ii) Immunohistochemical analysis - several attempts have been made to visualize histaminergic neurons in brain. Wilcox and Seybold (1982), using a fluorescently tagged antibody raised against a HA-methylated bovine serum complex, visualized neuronal cell bodies in the rat posterior hypothalamus. Fibers were seen in numerous areas, including several hypothalamic areas and the median eminence, the amygdala, hippocampus and to the cortex. No mention was made of any histaminergic innervation of the thalamus and the telencephalon was deemed to have the lowest fiber density of all areas examined. Of interest was the author's attempt to mask any interference by brain MCs by chronic (3 day) intraperitoneal administration of 48/80. No mention was made of the difficulty 48/80 has in penetrating the blood-brain barrier (see Discussion section VI) nor was there any mention of the visualization of MCs with this serum, either in the CNS or with peripheral MC-containing tissue that might act as a control. It was assumed by the authors that since 48/80 treatment did not affect the staining pattern of their specimens, any MC HA that might exist did not interfere with their study and that all the HA visualized was of neuronal origin.

A similar study was reported recently which in many ways confirms the above findings (Panula et al., 1984). Whereas Wilcox and Seybold (1982) reported that there was only one concentration of HA-containing nerve cell bodies in the brain, Panula et al. (1984) reported several areas of concentration, all within the posterior basal hypothalamus. Groups of cells were seen in the caudal magnocellular nucleus, anterior basal hypothalamus in the area of the ventral premammillary nucleus, in the lateral mammillary nucleus and around the dorsal tip of the third ventricle. Fibers were seen in cortex, hippocampus, basal hypothalamic nuclei and in several midbrain and pontine areas. Again, no mention was made of reactive cells or fibers in the thalamus. The authors were able to visualize MCs in several peripheral areas and found intensely staining MCs in the median eminence; unlike Wilcox and Seybold, this group (Panula et al., 1984) found few if any fibers in this area. This visualization of MCs in the median eminence is the first direct observation of these cells in this area as no anatomical reports of brain MCs have identified them there using classical histochemical methods. However, the presence of MCs in median eminence was hypothesized after biochemical evidence pointed to a non-neuronal source of HA in this nucleus with characteristics similar to those of peripheral MCs (a high HA level accompanied by a low HD activity, Pollard et al., 1976). There was no mention made of the visualization of MCs in thalamus (Panula et al., 1984).

Results similar to that of Panula et al. (1984) were obtained by Steinbusch and Mulder (1985), who used HA coupled to either bovine serum albumin or thyroglobulin. Unlike previous studies, a few fibers were observed in thalamus, primarily in the periventricular areas. Although no mention was made of thalamic MCs, these authors were able to observe brain MCs on the ventral surface of the hypothalamus.

A different approach to the problem of immunohistochemical visualization of histaminergic neurons was taken by Watanabe et al. (1983,1984). Instead of raising antibodies to HA, this group used the antigenic determinants of the enzyme histidine decarboxylase (HD) as their probe. These results were similar to the studies mentioned above. Neuronal cell bodies were confined to several areas within hypothalamus, namely the tuberal and caudal magnocellular nuclei, the posterior hypothalamic nuclei and in the lateral hypothalamus. Similar to the previous studies, fibers were found throughout the brain, with moderate to large numbers being located in cortex (diffusely), amygdala, basal ganglia and throughout the basal portion of posterior hypothalamus. A few fibers were visualized in thalamus, and these were confined to periventricular areas and the lateral geniculate. Few fibers were observed in brain stem. Although this group was able to visualize MCs in the rat peritoneal cavity with their serum (1983), no mention was made of any such observations in brain. Transection of the brain rostral to the posterior hypothalamus resulted in the loss of HD-

reactive fibers in cortex (Takeda et al., 1984), confirming the presence of neuronal cell bodies in posterior hypothalamus.

Thus, immunohistocytochemistry indicates that the posterior basal hypothalamus contains several groups of histaminergic cell bodies that project to diffuse areas of the brain. Thalamus is very sparsely innervated. Brain MCs (with the exception of those in median eminence) were not visualized with these methods. In none of these studies was any attempt made to identify brain MCs with standard histochemical techniques.

iii) Chemical analysis - while HA levels in different regions of rat brain varies between reports, all reports show similar regional patterns, with cerebellum having the lowest levels and hypothalamus the highest (Table 1). Whole brain HA values have been determined to be 40 to 60 ng/g in the adult rat (see Green, 1970).

C) Biochemical characterization of brain HA

i) Subcellular distribution, ontogeny and turnover of rat brain HA - subcellular fractionation of brain tissue has proved useful in examining the localization of several potential transmitter substances. Following homogenization of brain tissue in sucrose, the suspension is differentially centrifuged in density gradients to separate various cell compartments and organelle populations (see reviews: Marchbanks, 1975; Lahue, 1977). In adult brain, most of the endogenous HA is recovered in the P_2 crude mitochondrial

Table 1. Regional distribution of HA in adult male Sprague-Dawley rat brain.

	Authors							
Region	1*	2**	3**	4*	5	6	7	8
Cerebell.	19	8	4	22	26	22	11	26
Med-Pons	30	20	21	29	25	24	13	20
Midbrain	---	39	39	55	75	57	19	78
Thalamus	158	122	209	--		106	37	80
Hypothal	240	474	503	194	209	177	142	155
Cortex	39	26	38	39	39	57	13+	42
Striatum	56	28	32	78	56	49	23	50
Hippocam	40	18	16	33	50	38	15	31

Values (mean) of HA content (ng/g wet tissue weight) were rounded off to the nearest whole value. All studies were performed using the radioenzymatic assay for histamine except where noted. Both Schwartz et al. (1970) and Hough and Domino (1979) presented their data in graphical form. Taylor and Snyder (1972) presented a combined thalamus-midbrain analysis. The authors are as follows: 1) Blanco et al. (1973), 2) Oishi et al. (1983), 3) Oishi et al. (1984), 4) Schwartz et al. (1970), 5) Taylor and Snyder (1972), 6) Taylor and Snyder (1971), 7) Hough et al. (1984a), 8) Hough and Domino (1979).
Ages of animals and sacrifice methods were different between the studies.

*Fluorometric analysis with column purification
**HPLC and column separation of HA, and fluorometric analysis
+Frontal cortex only

subcellular fraction (11,500 g x 20 min; Marchbanks, 1975), which contains the majority of the synaptosomes (Table 2), indicating that brain HA is contained within nerve ending particles. However, there are significant levels in the P_1 crude nuclear pellet (900 g x 10 min; Marchbanks, 1975), indicating another possible localization of HA in a much denser particle than that found in P_2. Picatoste et al. (1977) added MCs isolated from rat peritoneal fluid to brain tissue prior to homogenization and found that almost all of the MC HA sedimented in the P_1 fraction, giving rise to the possibility that the HA found in this fraction was of MC origin. Young et al. (1971), aware of the possibility, microscopically examined the P_1 fraction from neonatal rats for signs of MC granules and failed to find any. However, it would take very few granules to account for the HA content of this fraction. Further, the methods by which the examination was made were not specified and unless great care was taken, it is quite possible that any MC granules present were not visualized.

In contrast to adults, HA in neonatal animals is found primarily in the P_1 subcellular fraction (Table 2). As neonates contain numerous MCs and MC HA sediments in this portion, this observation fueled speculation that P_1 HA might be indicative of a MC component in both neonates and adults. Following day 10, there is a drop in P_1 HA and a concomitant rise in P_2, reaching adult levels around day 21 (Young et al., 1971; Ferrer et al., 1979; Hough et al., 1982). The

Table 2. Comparison of the subcellular distribution of brain HA from neonatal and adult rats.

Author	Weight/ Age	Percentage of total HA P1	P2	P3	S
Neonates					
Young et al., 1971[1]	3 dys	91	9	0	0
Martres et al., 1975[2]	6 dys	80	--	--	10
Picatoste et al., 1977[2]	5 dys	86	9	1	3
Ferrer et al., 1979[2]	1 dy	84	[.....16.....]		
	5 dys	82	[.....18.....]		
Hough et al., 1982[2]	1 dy	70	20	5	5
Adults					
Carlini and Green[2]	------	18	18	44	20
Kataoka and DeRobertis[5]	------	8	21	51	20
Young et al., 1971[1]	21 dys	23	30	3	44
Snyder et al., 1974[3]	150-200 g	20	46	16	18
Picatoste et al., 1977[2]	150-250 g	28	39	11	22
Barbin et al., 1977[4]	------	32	37	13	--
Ferrer et al., 1979[2]	60 dys	20	[.....80.....]		
Hough et al., 1982[2]	105 dys	18	32	13	38

Shown are the mean values for percentage of total brain HA per subcellular fraction. Values were rounded off to the nearest whole number where required. With the exception of Martres et al. (1975), who counted CPM per fraction following injection of ^{3}H-histidine, all studies measured endogenous HA.

[1] only diencephalon assayed
[2] whole brain assayed
[3] only hypothalamus assayed
[4] measured control cortex contralateral to lesioned hypothalamus
[5] only cortex assayed

activity of histidine decarboxylase (HD), which is low in neonates, increases at day 10 to reach adult levels at a similar time as the P_2 HA (Schwartz et al., 1971; Martres et al., 1975). This enzymatic activity is also confined to the P_2 subcellular fraction (Baudry et al., 1973; Snyder et al., 1974). Such observations led to the expanded hypothesis that the primary source of HA in neonates is the MC and that as the animal matures, the contribution of the histaminergic neuronal system (which is undeveloped at birth) increases to reach adult levels around day 21. This is consistent with the ontogeny of the changes in rat brain MC number and distribution (see Introduction section II-D).

Further evidence supporting this hypothesis (that MCs account for most of neonatal brain HA, but that this contribution declines relative to neurons as the animal matures) was found in the analysis of HA turnover. HD activity is low in neonates, leading to a high HA/HD ratio which is similar to that found in peripheral MCs (Martres et al., 1975). The half-life ($t_{1/2}$) of this amine in neonates is longer (5 days: Martres, 1975) than that found in adults ($t_{1/2}$<1 hour: see review by Hough and Green, 1984). In addition, intra-cranial injection of compound 48/80 into neonates produced a significant drop in the levels of HA in the P_1 fraction while no change was observed in P_2 HA (Blanco et al., 1977). This drop was assumed to be due to the degranulation of brain MCs, which has been confirmed histochemically (Persinger, 1983).

Taken together, this evidence suggests that the bulk of HA in neonates is of MC origin and may be in the P_1 fraction. However, more work needs to be done before any theories can be postulated. With the exception of Ferrer et al. (1979), none of the investigators examining the subcellular distribution of HA in neonates or adults physically counted brain MCs. Ferrer et al. (1979) postulated that the 23 to 30% of total adult brain HA found in the P_1 fraction was contributed by MCs. However, using his values for MC numbers and whole brain HA content, the HA content of adult (60 dys) brain MCs would be 14.3 to 18.6 pg HA per MC. It is unlikely that brain MCs contain HA levels of this nature, as less than 400 MCs of this type would be able to account for the entire thalamic HA content and various authors have indicated that adult rat thalamus contains several times this number (Section II). Further, MC numbers vary widely between individuals, yet estimates for the portion of brain HA found in P_1 show less variation or such data are not presented. Although adult brain MCs are confined to thalamus, none of the subcellular studies evaluated the distribution of thalamus relative to other brain regions. Thalamus might be expected to have a different fractional pattern (higher P_1/P_2) than hypothalamus, cortex or cerebellum, but this has not been studied. Finally, the ontogenic pattern of tMH is similar to that of HA. Correlations of HA versus tMH are significant in most subcellular fractions from day 4 on (Hough et al., 1982). Should brain MCs be similar to

peripheral MCs (which contain little tMH: Goldschmidt et al., 1984a), the ontogenic pattern of tMH would be expected to be different from that of HA. While this argues against the hypothesis that all neonatal HA is from MCs and that these cells are the same as those found in the peripheral tissues of adults, it is possible that neonatal brain MCs turn over HA more rapidly than is found in adults, and that as the animal matures, MC turnover of HA becomes less rapid and the tMH present arises from other sources, e.g. (neurons).

In summary, although subcellular distribution studies on the ontogeny of HA and examination of the patterns of brain MC numbers and distribution tend to support the concept that much of the HA in neonatal rat brain is from MCs, the subcellular evidence for MC contribution to adult brain HA is more equivocal.

ii) Brain lesions and the effects on HA and HD activity in areas distal to the lesion site - based on the concept that discrete brain lesions would cause disruption of histaminergic fibers and result in a decrease in HA and HD activity in regions innervated by these fibers (thus tracing the course of the histaminergic system), selected lesions were placed through the median forebrain bundle in lateral hypothalamus, and cortical HA levels and HD activity monitored (Garbarg et al., 1976; Barbin et al., 1977). HA levels and HD activity decreased in the ipsilateral cortex by approximately 25% and 50% respectively relative to contralateral control cortex, indicating that a certain

portion of cortical HA was sensitive to such ablations and might be of neuronal origin. However, a significant portion of cortical HA was resistant to such treatment, suggesting a non-neuronal pool of HA within cortex. The timecourse of the decreases of HA and HD activity was consistent with that of Wallerian degeneration (MacDonald et al., 1981).

These changes in HD and HA were shown to be the result of the disruption of a histaminergic pathway and not a general phenomenon as administration of 6-hydroxydopamine and 5,6-dihydroxytryptamine, which lead to selective destruction of catecholamine and 5HT neurons respectively, resulted in small changes in cortical HA and HD activity while resulting in large decreases in noradrenaline and 5HT. Of particular interest was the finding that the decrease in HA following lesions was due solely to a decrease in the P_2 content and that P_1 HA was unchanged (Barbin et al, 1977). The sub-cellular profile of HD was not examined, but normally it is found in the P_2 fraction (Baudry et al., 1973). These data were taken as evidence of a MC component to cortical HA amounting to approximately 50% of the total amine present. Similar lesions established that the cell bodies of the neurons projecting to rostral areas resided in the posterior hypothalamus (Garbarg et al., 1976,1980), an assertion recently confirmed through the use of immunohistochemistry (see above).

While lesion studies have provided good evidence for neuronal and non-neuronal HA in rat cortex, the association

of this non-neuronal HA with MCs cannot be maintained. Anatomical studies have found few MCs in cortical areas of the adult rat. Further, MCs increase in numbers in regions distal to sectioned peripheral nerve (Enerback et al., 1965; Olsson, 1968; MacDonald et al., 1981) and such a phenomenon might raise post-lesion HA levels in cortex higher than they might otherwise be. Thus, the effects of lesions, while strongly indicating the presence of neuronal and non-neuronal HA in cortex, cannot tell us anything about the presence of brain MCs in cortex.

iii) Effects of the inhibition of HD - Inhibitors of HD have been used to characterize neuronal HA. The rationale for these studies has been that, unlike MCs, neurons are a source HA with a rapid turnover and that inhibition of the synthetic enzyme would result in an immediate decrease of this amine in neurons. Such was observed, as peripheral administration of the HD inhibitors alpha-hydrazinohistidine and 4-bromo-3-hydroxybenzyloxamine (NSD-1055)(200 mg/kg I.P.) resulted in a drop in rat brain HA that was maximally decreased within 30 min and returned to normal levels within 24 hours (Taylor and Snyder, 1971). Interestingly, the greatest decrease in HA levels occurred in the thalamic-midbrain area (approximately 45%), while levels in hypothalamus and cortex were lowered by about 35% and 22% respectively. Similar results were obtained with the use of the suicide inhibitor of HD, alpha-fluoromethylhistidine. Brain HA levels were lowered in rat whole brain (Bouchlier et

al., 1983) by approximately 50%. The drug lowered HA levels by a similar amount in mouse cortex (Garbarg et al., 1980b) and mouse whole brain (Maeyama et al., 1982). Interestingly, this inhibitor lowered HA levels in W/W^{v} mice (mutants deficient in MCs, see section III-C-v) almost completely, while HA in normal control mice (+/+) was lowered by 50% (Maeyama et al., 1983). These observations have led to the hypothesis that MCs are responsible for the portion of brain HA that is resistant to the effects of HD inhibition, i.e., that 50% of brain HA is neuronal and the remaining 50% is due to brain MCs.

Similar criticisms can be made of these studies as those made of the lesion experiments. While there is good evidence to support the concept of neuronal and non-neuronal HA, to link this non-neuronal HA to MCs is premature, especially as decreases in HA have been found to be larger in rat thalamus (which contains most of the MCs) than in hypothalamus (which contains few or no MCs but all the histaminergic cell bodies). That 50% of rat cortical HA (and cortex contains few MCs) and of mouse brain HA (mouse brain has few MCs relative to rat, see section III-C-v) is resistant to these drugs is further evidence that this non-neuronal HA is not primarily due to MCs.

iv) Release studies - several studies have attempted to characterize the release of neuronal HA and have also found evidence for non-neuronal pools. 48/80 caused a significant release of HA from adult rat hypothalamic slices in the area

of the median eminence, an area that also had a $^{HA}/_{HD}$ ratio similar to that of peripheral MCs (Pollard et al., 1976). ^{3}H-HA was released by potassium depolarization from hypothalamic slices preincubated with ^{3}H-histidine (Verdiere et al., 1975) or ^{3}H-HA (Subramanian and Mulder, 1976) in a Ca^{+2} dependent manner, a mechanism resembling neuronal release. 48/80 also released HA from these preparations. Further, based on the specific activity of the HA released by the two methods, it was determined that the HA released by 48/80 was from a slowly turning over pool (low label) while that released by depolarization was in a pool that turned over quickly (Verdiere et al., 1975). In addition, the time course of the release by 48/80 was much slower than that of potassium (Subramanian and Mulder, 1976). The K^{+}-released HA and that released by 48/80 were independant of each other, i.e., release of one pool had no effect on the other pool (Verdiere et al., 1975). Taken together, these data have suggested the presence of two pools of histamine resident in brain: neurons and MCs. However, in none of these studies was any attempt made to identify MCs using histological techniques.

Due to its high HA levels, the preponderance of these studies have concentrated on hypothalamus and none of these studies have characterized thalamic HA. Thus, there is no direct evidence linking 48/80 release with MCs. It would be interesting to combine release studies on rat thalamus with subcellular analysis and histological evaluation to provide

direct linkage of MCs identified with classical methods with the types of biochemical reports that have been published to date.

v) Studies with the mutant mast cell-deficient mouse - the W/W^v mouse is a mutant with a deficiency in the pluripotential hematopoetic stem cell that gives rise to serosal MCs (Kitamura and Hatanaka, 1978). While peripheral organs contain only 2% of their normal MC numbers (Kitamura and Hatanaka, 1978; Watanabe et al., 1980), the brain seems to be completely devoid of these cells (Hough et al., 1984). Thus, these animals would seem to be an ideal vehicle for the study of the contribution of brain MCs to brain HA in a mammalian species. Indeed, HA levels in the brains of the mutant W/W^v strain have been reported to be approximately half of that of their normal +/+ control counterpart (Yamatodani et al., 1982; Grzanna and Schultz, 1982; Maeyama et al., 1983). While the HA levels in +/+ mice evaluated by Yamatodani et al. (1982) were similar to those found in other studies (53 ng/g), those found by Grzanna and Schultz (1982) were significantly higher (5-10 x) in all brain regions, allowing for the possibility that their results might be artifactual. As the number of MCs in the brains of +/+ controls are low compared to that of rats (Orr and Pace, 1984; Hough et al., 1984), MCs in the brains of these animals would have to contain very high levels of HA to make any significant contribution to the overall HA pool. Other studies have failed to confirm differences in brain HA levels

(Orr and Pace, 1984; Hough et al., 1984) or brain tMH levels (Hough et al., 1984) between +/+ and W/W^v mice. Subcellular fractionation of +/+ and W/W^v brains shows that the mutant animals have a larger percentage of their brain HA in the P_1 fraction, the opposite of what might be expected (Orr and Pace, 1984). The use of alpha-fluoromethylhistidine, an inhibitor of HD that has been used to characterize neuronal HA in rat brain (see above), lowered the HA content of W/W^v mouse brain by the same amount (50%) as it did in normal rat brain (Orr and Pace, 1984), indicating the presence of a non-neuronal pool of HA in the brains of animals that lack MCs. In contrast, this substance was reported to have lowered the HA content in mutants almost completely, while controls were lowered by 50% (Maeyama et al., 1983). The stem cell defect that results in the lack of MCs also has numerous effects on other hematopoetic cells (Kitamura and Hatanaka, 1978; Geissler and Russell, 1983), leaving open the possibility that any difference in tissue HA between mutant and control might be due not only to MCs but to other cell systems as well.

D) Attempts to estimate the contribution of brain MCs to brain HA based on anatomical evaluation of brain MC numbers

Histological methods have been used to estimate the MC contribution to brain HA levels. Dropp, assuming a HA content of 20 pg/MC (the levels in peritoneal MCs), suggested that 7-29% (1972) or 68% (1976) of rat brain HA could be of MC origin. However, these estimates were based on brain HA

levels of 200-400 ng/g, values much higher than those presently accepted (40-50 ng/g; Green, 1970; Hough and Green, 1984). Persinger (1977) speculated that MCs could contribute 29-100% of the HA in rat brain. Similarly, Kruger (1974) speculated that most of the HA in adult thalamus could be of MC origin. Of interest is the wide range of possible HA contributions that these studies attribute to MCs; this is probably a reflection of the wide range in brain MC numbers between animals.

These estimates of the contribution of brain MCs to brain HA have been flawed by the use of two assumptions: 1) that the HA content of rat brain MCs is the same as that of peripheral (peritoneal) MCs and 2) that brain HA content or brain MC content is the same in every animal. No evidence has been published to indicate that brain MCs contain HA and no studies have been able to establish an acceptable value for the HA content of these cells. Indeed, it is unlikely that brain MCs would contain similar levels as their counterparts in the periphery as only 300 thalamic MCs would be required to account for the entire thalamic HA content (assuming: 20 pg HA/MC, Goldschmidt et al., 1984a; thalamic HA level=100 ng/g, see Table 1; thalamic weight=0.06 g). As discussed (Introduction section II), adult rat thalamus has many more MCs than this value.

Should brain MCs contain considerable HA, the variability in MC numbers might be expected to result in a large variability in thalamic HA and a smaller (though still

significant) variability in whole brain HA. With the exception of the studies of Oishi et al. (1983,1984), this has not been observed. At least three possibilities exist to reconcile these facts: 1) either brain MCs contain lower levels of HA than is contained in peripheral MCs, 2) studies to date have used methods that do not measure the HA contained within brain MCs or 3) brain MCs contain no histamine and the non-thalamic HA observed by the various biochemical studies described above was from a different, non-MC cell population.

IV) Brain tMH - relationship to neurons and MCs

Within the CNS, HA is primarily if not exclusively metabolized to tMH (section III-A). The regional distribution of tMH and HA are similar (Hough and Domino, 1979) and the levels of these two amines are equivalent in many brain areas (Hough and Domino, 1979; Oishi et al., 1983; Oishi et al., 1984; Hough et al., 1984a). Neuronal HA turnover is rapid (section III-C-i), and inhibition of HMT leads to a fast rise in brain HA (see Hough and Green, 1984). These findings have prompted investigators to hypothesize that tMH might be an indicator of histaminergic neuronal activity (Oishi et al., 1983; Oishi et al., 1984; Hough et al., 1984a). This hypothesis, however, assumes that the only source of brain tMH is neuronal HA.

As discussed, there might be sources of HA in the CNS other than neurons (i.e., MCs). Should any of these sources contribute to brain tMH, the measurement of this amine as an

index of neuronal HA would not be appropriate. While peripheral MCs contain high levels of HA, the turnover is slow and the cells contain only insignificant levels of tMH (section I-D-i). The levels of HA and tMH in brain MCs are not known, and the turnover of HA in these cells has not been established. Therefore, a major goal of this thesis was to investigate whether brain MCs contribute to brain tMH, as well as to HA.

V) Summary of the literature relating to brain MCs and brain HA and listing of the goals of this thesis

In conclusion, little is known about brain MCs and less is known about how these cells might contribute to brain HA levels. Although they have been characterized with several histological stains, the precise methods used have often been inadequate or have not been clearly presented. There have been no studies to indicate that the same population of cells binds all the dyes that have been used under conditions most specific for MCs.

Although several studies have indicated that these cells are found in large quantities in the thalamus, almost no quantitative data are available concerning the precise contribution that rat thalamus makes to the total number of MCs in brain and little data exist concerning MC numbers in other brain regions. Almost nothing is known about the distribution of MCs within thalamus. Although brain MCs show considerable variability in numbers between individuals, most studies that have attempted to quantify them have used too few animals and have not been sufficiently rigorous.

Although anatomical studies have estimated the contribution of brain MCs to brain HA, and biochemical studies have inferred the presence of MCs based on brain HA that has different characteristics from that of neuronal HA, no direct evidence has been presented linking brain MCs to brain HA. Indeed, there is no biochemical evidence whatsoever that brain MCs contain HA at all.

In an attempt to rectify some of these problems, experiments were designed to meet several specific goals:

1) To use specific histochemical techniques to characterize brain MCs under conditions that show unequivocally that they have a similar histological profile as peripheral MCs.

2) To use the gaseous OPT technique to provide direct evidence that these cells contain HA and show that these HA-containing cells are MCs.

3) To quantify rigorously brain MCs both as a function of whole brain distribution and as a function of the anterior-posterior and nuclear distributions within thalamus.

4) To use a combination of histochemical and biochemical techniques to directly study the relationship between brain MCs, brain HA, and it's metabolite tMH.

Materials: Sprague-Dawley rats from Perfection Breeders (Douglasville, PA); dichlorodifluoromethane (freon, UCON 12) from TW Smith (New York, NY); toluidine blue O, histamine dihydrochloride, S-adenosylmethionine iodide - Grade 1, Compound 48/80 and ortho-phthalaldehyde from Sigma Chemical Co. (St. Louis, MO); paraformaldehyde from Fisher Scientific Corp. (Fairlawn, NJ); glutaraldehyde from Kodak Co. (Rochester, NY); Astrablau FM from Roboz Surgical Co. (Washington, D.C.); heptafluorobutyric anhydride and Reactivials from Pierce Chemical Co. (Rockford, IL); 3-methylhistamine (tele-methylhistamine) and 1-methylhistamine (pros-methylhistamine) from Calbiochem (LaJolla, CA); 3% Poly I-110 in Gas Chrom Q, 80-100 mesh from Applied Sciences Laboratories; ^{3}H-S-adenosylmethionine (NET-155H, 55-85 Ci/mmol, in sulfuric acid: ethanol (9:1), pH 2.0, >98% pure) from New England Nuclear (Boston, MA).

I) Characterization of rat brain MCs

A) Toluidine blue histology

i) Preparation of brain tissue and cryostat techniques - adult albino Sprague-Dawley rats (250 to 300 g) were killed by decapitation. Brains were dissected free of dura and quickly removed from the cranial case onto a wetted counter top with the ventral surface down. A chilled (in liquid dichlorodifluoromethane; freon) glass microscope slide was touched to the dorsal surface of the brain, freezing the tissue onto the slide. The slide with the brain attached was

immersed in liquid freon for 30 sec and then thaw-removed from the slide. Following a quick re-freezing in liquid freon, the brains were placed in a Damon-Minotome microtome cryostat (-16°C). A small portion of the posterior brain stem was trimmed flat with a chilled razor blade and the brain mounted onto a chilled cryostat chuck with distilled water at the posterior end. Following positioning of the brain so that its angle corresponded to that of the brain atlas used (Pellegrino et al., 1979), 10 micron (um) sections were taken and thaw-mounted onto room temperature glass slides. Tongues were also sectioned as they contain numerous mast cells; sections of brain and tongue were always processed on the same slide for comparison and as a check on staining procedures.

ii) Fixing and staining of tissue sections - to characterize brain MCs, sections were fixed in chilled (4°C) 80% ethanol: 30% formaldehyde: glacial acetic acid, 90:5:5 (v/v/v, EFA) containing 1% glutaraldehyde for 5 min and then washed with 3 consecutive 1 min water washes. Following fixation, slides were stained in either 0.25% aqueous toluidine blue (pH 3.3) or in 0.25% aqueous toluidine blue further acidified with HCl to pH 2.3.

1) 0.25% aqueous toluidine blue (pH 3.3) - slides were immersed in stain for 1 min and then washed in 5 consecutive water washes of various durations:

wash 1: 15 sec with an agitation after 7 sec.

wash 2: 15 sec with an agitation after 7 sec.

wash 3: 30 sec with an agitation after 15 sec.

wash 4: 1 min with an agitation after 30 sec.

wash 5: 1 min with an agitation after 30 sec.

Slides were decolorized with graded ethanol washes in order to decrease background staining and to bring the tissue to anhydrous conditions needed for cover-slipping:

wash 1: 70% ethanol for 90 to 105 sec with agitation every 15 sec.

wash 2: 95% ethanol for 30 sec with agitation after 15 sec.

wash 3: 100% ethanol for 30 sec with agitation after 15 sec.

wash 4: absolute xylene for 10 min with agitation every 2 to 3 min.

Following xylene, the sections were cover-slipped with Permount under glass cover slips.

2) 0.25% acidified aqueous toluidine blue (pH 2.3) - slides were immersed in stain for 10 min and then washed in 3 consecutive 2 min aqueous acidified (HCl, pH 2.5) washes. Slides were decolorized and dehydrated in graded ethanol:

wash 1: 70% acidified (HCl, pH 2.5) ethanol for 1 min with agitation every 15 sec.

wash 2: 95% ethanol for 1 min with agitation every 15 sec.

wash 3: 100% ethanol for 1 min with agitation every 15 sec.

wash 4: absolute xylene for 10 min.

Slides were cover-slipped as described above.

B) Astrablau histology and comparison with toluidine blue - fresh cryostat sections (obtained with the same techniques as outlined above) were fixed and stained simultaneously in 0.005% toluidine blue in 50% ethanol (pH 7.1) for approximately 5 min. Following photography, the sections were destained in 70% acidified (HCl) ethanol (pH 3) for 10 min, restained with 0.05% Astrablau in 50% acidified (HCl) ethanol (pH 1) overnight, brought to anhydrous conditions with graded ethanols and xylene, cover-slipped with Permount, and rephotographed. EFA fixation was omitted because aldehydes interfere with Astrablau binding (Padawer, 1966).

C) Ortho-phthalaldehyde (OPT) histochemistry - the HA content of rat brain MCs was evaluated with a modification of the gaseous OPT method (Ehinger and Thundberg, 1967). Fresh cryostat sections (obtained as described above) were cut at -18°C and mounted onto pre-chilled glass slides (liquid freon) by pressing the slides onto the sections as they lay on the cryostat knife. Slides were transferred to horizontal slide racks and the racks placed in chilled glass containers for freezer storage while the remainder of the brain sections were obtained. Sections were dessicated overnight in a Virtis freeze dryer at -50°C and 10 millitorr. After warming to room temperature, sections were removed from the dryer and stored in a glass dessicating chamber over phosphorus pentoxide at room temperature until use. Sections were removed from the dessicating chamber and briefly exposed to

hot acetone vapor, which bonded them firmly to the glass slides. The slides were exposed to OPT for 15 sec by placement in a preheated (110°C, in oven) sealed 3 l steel vapor chamber previously equilibrated for 10 min with 150 mg OPT. Sections were then exposed to gentle steam for about 3 sec, and mounted in tetrahydrofurfuryl alcohol (THF-OH). Appropriate fields were photographed (Zeiss microscope with HBO-200 UV light source, UG-1 and 530 nm filters, and Kodak Tri-X film, ASA 400).

In some experiments, the same brain sections were examined for both OPT and toluidine blue reactivity; fluorescent MCs prepared and photographed as described above were stained under the coverslip with toluidine blue without moving the slide. This was accomplished by touching one side of the cover slip with a piece of paper towel to draw out the THF-OH while introducing 0.005% toluidine blue in 50% ethanol dropwise to the other side of the cover slip. The sections were then re-photographed with the same film, but with white light (normal incandescent low voltage bulb) and no filters.

D) Paraformaldehyde (PF) histochemistry - the gaseous PF method was used (Falck et al., 1962) as modified by Hamberger and Norberg (1964), with suggestions by J. Padawer (personal communication). Brain cryostat sections were obtained and acetone bonded onto glass slides as described above in the OPT method. Sections were placed in a glass jar that had a sealable screw top lid. PF (0.75 g) was placed in a small concave petri dish, 3 drops of distilled water placed on the

PF, and the petri dish placed inside the glass jar under the glass tissue slides. The entire jar was sealed and placed in preheated oven (100°C) and the oven temperature decreased to 80°C. After 1 hr, the jar was removed from the oven, allowed to cool, opened, and the treated slides placed inside a light-proof plastic slide box (the treated slides were not allowed to be exposed to fluorescent light following their removal from the treatment jar). Slides were examined with the Zeiss fluorescent microscope described above with BG-12 and 530 nm filters in place. Photography of fluorescent tissue sections was performed as described above.

Following fluorescent photography, slides were exposed to gentle steam for 10 sec and allowed to dry for 2 min in order to bond tissue to glass slides. In the absence of steam treatment, exposure to aqueous stains causes such extensive rippling of tissue as to render them unusable. Sections were restained with 0.01% toluidine blue in 70% ethanol (approximately 3 min) and rephotographed.

II) Quantification of brain MCs

To quantify brain MCs, serial 10 u cryostat sections were taken every 0.18 to 0.20 mm and were fixed and stained in 0.25% aqueous toluidine blue (pH 3.3) as described above. The entire section was scanned (x100) with an ocular grid to ensure that no areas of the tissue were scanned twice. Only MCs with diameters greater than 8 um (to eliminate cell fragments) and containing distinct metachromatic granules

were counted. The anterior-posterior (A-P) coordinate of each section (in mm from bregma, which is 0), and the nuclear localization of each cell (Pellegrino et. al., 1979) were noted. Re-counting of coded slides yielded results within 10% of the original counts. Whole brain MC values were obtained by multiplying slide counts by 20 to account for the volume of tissue actually sampled. Total MCs per thalamic nucleus can likewise be obtained by multiplying total observed MC numbers per nucleus (Table 6) by 20.

III) Preparation of tissue homogenates and sections for analysis of MC numbers, HA and tMH levels from the same brain

A) Tissue dissection and slide preparation - whole brains from decapitated Sprague-Dawley rats (female: 225-285g; male: 230-365g) were frozen for 1 min in liquid freon. Frozen brains were placed on a pre-chilled lucite inclined plane (angle= 19°) with the dorsal and anterior surfaces down (Herberg and Franklin, 1973), and a single coronal cut with a chilled razor blade was made through the diencephalon aimed at the level of the lateral habenular nucleus. Only brains that had been cut between coordinate values -0.4 mm and -3.0 mm (anterior thalamus = +1.0 mm; posterior thalamus = -3.6 mm) and whose lateral coordinates (left and right sides) were not more than 0.2 mm apart were retained for further processing. A small portion of the brain stem and cerebellum was trimmed flat and the posterior brain half was mounted with deionized water in the cryostat at -16°C. The anterior

half was temporarily stored in a freezer at the same temperature. Following adjustment of the brain and several preliminary slices to ensure an even and complete section, 2 consecutive 10 um sections were taken and thaw mounted onto glass slides. In no case was more than 70 um of tissue taken (about 1.5% of thalamic volume). All brain tissue remained frozen throughout the procedure. The cryostat sections were fixed and stained for MCs using the aqueous 0.25% toluidine blue (pH 3.3) method described above. MC numbers were determined for left and right sides from the first of the 2 consecutive sections; coordinates were identified from either or both sections. Blinded recounts yielded results within 10% of the original values.

B) Estimation of thalamic MC numbers - total thalamic MC numbers for each side of brain were estimated by dividing the MC number from each side of a single section by the slope of the appropriate correlation curve relating MCs/section for that coordinate to MCs/thalamus (see Results, Table 7,8 and Fig 10). Total predicted thalamic MC numbers were the sum of the calculated MC numbers from left and right sides multiplied by 20.

C) Homogenization of frozen brain tissue and preparation of samples for biochemical analysis - the posterior brain half was removed from the cryostat chuck and cleared of mounting ice. Brain halves were placed on dry ice, bisected sagittaly along the midline with a chilled razor blade, and the thalamus dissected out of the frozen quarters. Thalamic

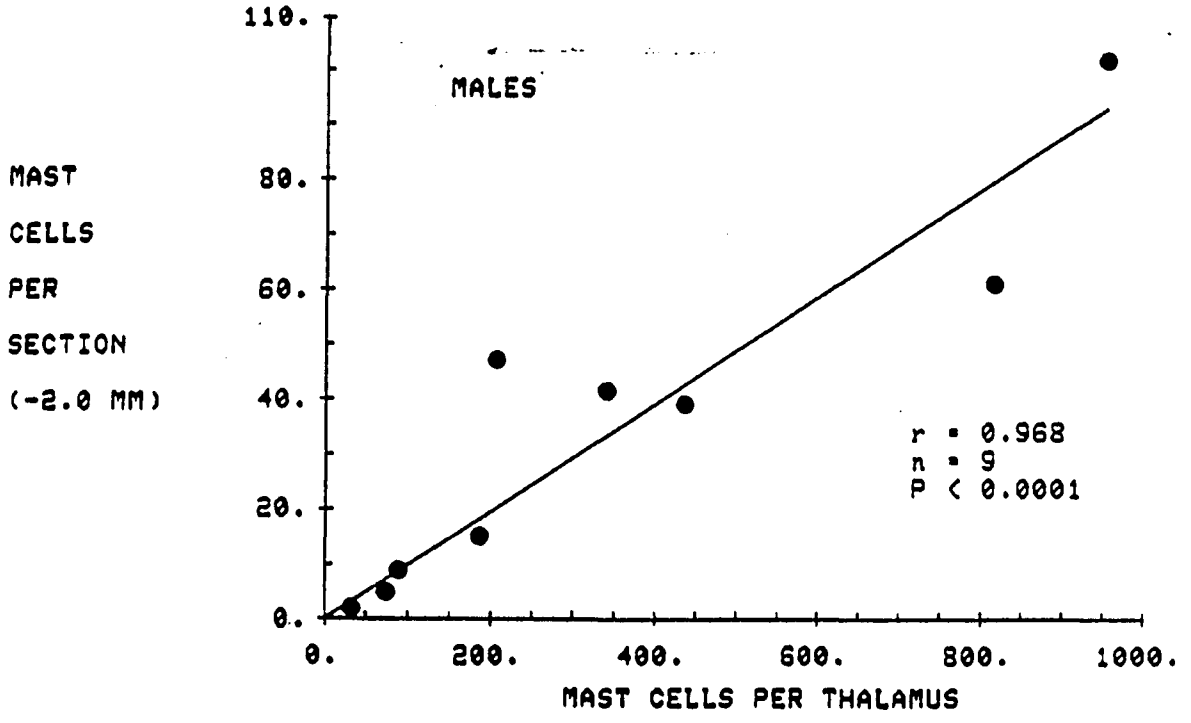

Fig 10. Correlation of thalamic MC numbers from single diencephalic coronal sections (-2.0 mm) with total MC numbers of male rat brains. Ten micron cryostat sections were taken through the diencephalon, and were counted and stained for MCs as described. The correlation was forced through zero.

and non-thalamic (i.e. the remainder of the brain) portions were weighed and homogenized in cold deionized water for 15 sec in a Polytron homogenizer (0.5 ml for thalamus tissue and 7.0 ml for the remainder of the brain). For HA analysis, 75 ul aliquots were diluted 1:2 with 0.1 M sodium phosphate buffer (pH 7.9) in 200 ul disposable polypropylene tubes, and heated in boiling water for 10 min, cooled on ice for 10 min and frozen until analysis. For tMH analysis, 75 ul aliquots were denatured with 75 ul of 0.8 N perchloric acid (in 200 ul polypropylene tubes), cooled for 10 min on ice and frozen until use.

IV) Preparation of tissue for analysis of HA from fresh and frozen brain tissue.

A) Fresh brain tissue - adult male Sprague-Dawley rats (body weight: mean±SEM = 317±7g; range: 295 to 355g) were decapitated and their brains removed onto a cool plate (on ice). Brains were immediately bisected sagittally along the midline and then cut into quarters through the mid-diencephalon. Brains were dissected and combined to yield 4 areas: thalamus, hypothalamus, cerebellum-brainstem, and the remainder (which included cortex, hippocampus, midbrain, caudate, etc). Tissues were immediately weighed and homogenized with distilled water for 15 sec in Polytron homogenizer: thalamus and hypothalamus were homogenized in 0.6 ml water, cerebellum-brainstem in 2.5 ml water, and the

remainder in 5.0 ml water. Homogenates were prepared for HA analysis as described above.

B) Frozen brain tissue - adult male Sprague-Dawley rats (body weight: mean±SEM = 302±6g; range: 270 to 315g) were decapitated and the brains frozen for 30 sec in liquid freon as described. Frozen brains were dissected on dry ice into 4 regions, homogenized and prepared for HA analysis as described above.

V) HA analysis

A) Preparation of histamine N-methyltransferase (HMT) - partially purified rat kidney HMT was obtained by the method of Shaff and Beaven (1979).

B) HA analysis - HA was assayed by the single isotope radioenzymatic assay, originally described by Taylor and Snyder (1972) and modified by Beaven et al. (1972) and Verburg et al. (1983). Endogenous HA is incubated in the presence of HMT and ^{3}H-S-adenosylmethionine (SAM, a methyl donor) to form ^{3}H-tMH. This isotopic tMH is extracted into chloroform, the chloroform transferred into counting vials and allowed to dry. The sample was resuspended in scintilation cocktail and counted.

Biological samples prepared as described above were centrifuged (20,000x g for 10 min) and the supernatant fractions assayed either undiluted or diluted (thalamus and hypothalamus; 1:3 or 1:4 dilutions) with 0.05 M phosphate buffer pH 7.9.

i) Preparation of HMT-SAM mixture - for 100 ul of biological sample to be assayed, 100 ul of ^{3}H-SAM (80 Ci/mol; 0.5 mCi/ml) was evaporated to dryness in a glass culture tube on ice under dry N_2 gas (approximately 1 hr). This was resuspended in 855 ul 0.05 M phosphate buffer pH 7.9, 45 ul 0.25 M non-isotopic SAM and 100 ul rat kidney HMT.

ii) Pipetting and incubation - 10 ul of biological sample were assayed with (10 ul 0.01 ng/ul HA standard in 0.05 M phosphate buffer) or without (10 ul 0.05 M phosphate buffer) exogenous HA internal standard. Incubation was performed in iced (4°C) 400 ul polyethylene disposable tubes. To each tube was added 10 ul of the HMT/SAM mixture described above. Tubes were incubated for 1 hr on ice and then stopped by addition of 0.8 N perchloric acid with 0.05 mg/ml tMH added as a carrier. All pipetting was performed with Rainin adjustable Pipettman pipettes.

iii) Extraction of sample and measurement of isotope - samples recieved 10 ul 10 N NaOH, follwed by 200 ul chloroform. The samples were mixed by vortex for 15 sec and centrifuged. The top aqueous layer was removed and 3 N NaOH (50 ul) added. Following mixing by vortex and centrifugation, the top layer was again removed and 100 ul of the chloroform pipetted into a counting minivial. The chloroform was allowed to evaporate to dryness at room temperature and 5 ml counting cocktail (NEN 955) added to the vials. After capping and shaking, the vials were loaded into a counter

(Beckman LS-250 or LS-3801) and measured over 2 cycles at 5 min per count.

Most recovery values were around 100% Samples having recoveries below 75% or above 120% were either re-assayed or discarded. All HA levels were corrected for recovery. Three homogenates out of 101 yielded HA values greater than 3.5 standard deviations from the sample mean, and values from these animals were rejected.

VI) Tele-methylhistamine (tMH) analysis

A) Preparation of samples and extraction - the method for analyzing brain samples for tMH has been described by Hough et al. (1981). The perchloric acid precipitated brain homogenates described above were centrifuged (20,000x g; 10 min) and the supernatants withdrawn (approximately 125 ul). These supernatants were diluted to 1 ml with 0.4 N perchloric acid and 15 ng pros-methylhistamine (pMH) added as internal standard. To each tube (15 ml conical polyethylene disposable) was added 0.2 ml 10 N KOH, a saturating amount of NaCl, and 5 ml of n butanol-chloroform (1:1). Following vortexing, the tubes were mechanically shaken for 30 min and then centrifuged (5000x g; 5 min) to separate the phases. Each upper organic layer was transferred to a second tube containing 0.5 ml of 0.01 N HCl and 5 ml of heptane, with care taken to prevent any accidental transfer of the bottom layer which would basify the subsequent extraction and destroy the procedure. The tubes were shaken for 15 min and

centrifuged as described. The upper organic layers were removed and the bottom aqueous layer transferred to silanized Reactivials. Care was taken to aspirate all the organic layer and the pH of the lower layer was checked with pH paper to ensure that this solution remained acidic. The samples were evaporated to dryness at room temperature in a Savant vacuum centrifuge apparatus, capped and stored until further processing.

B) Derivatization and extraction - the residues were resuspended in 30 ul of pyridine:toluene (1:5). The vials were capped, vortexed for 1 min, and centrifuged in the Savant apparatus for 1 min at atmospheric pressure. Each vial received 15 ul heptafluorobutyric anhydride (HFBA) to the samples. The vials were recapped, vortexed for 10 min intermittently, the reaction stopped by addition of 200 ul saturated tris buffer pH 8.0, and the vials centrifuged for 1 min. During the vortexing period, the vials were checked for the presence of 2 phases, indicative of reactive HFBA. After centrifugation, the lower aqueous phase was withdrawn through a syringe and discarded. Vials were again recapped, vortexed, centrifuged and the bulk of the remaining aqueous phase removed. Each vial received a small amount of anhydrous granular sodium sulfate to trap any remaining aqueous phase. Following vortexing, vials were centrifuged for 1 min and the supernatants analyzed.

C) Measurement of derivatized sample - GCMS analysis of tMH was performed by Dr. Jai Khandelwal (Mt. Sinai School of

Medicine, Deptartment of Pharmacology), to whom I am most grateful. Samples were injected into a 6 ft by 2 mm silanized glass column containing 3% Poly I-100 on a Gas Chrom Q, 80-100 mesh, with helium (20 ml/min) as carrier. Electron impact mass spectra were obtained on a Hewlett-Packard 5930A combined GCMS (70 eV electron energy, 0.3 mA emission). A Hewlett-Packard 5933A data system linked to a Tektronix 4012 terminal equipped with a model 4610 hard copy unit was used to acquire, reduce, and process the data including the determination of the peak areas of the selected ion scans. For quantification, selected ion scans of m/e 304 corresponding to both tMH and pMH were monitored. Standard curves, carried through the assay procedure, were constructed with each assay of biological samples. The plot of the ratios of the peak sizes of the derivatives of tMH and pMH (tMH/pMH) versus the amount of tMH yielded a standard curve that was linear from 1 to 25 ng (free base).

VII) Pharmacological characterization of rat brain MCs with compound 48/80

A) Intraperitoneal injection - female Sprague-Dawley rats (body weight=250 g) were injected with compound 48/80 in normal saline (0.9% NaCl). Doses ranged from 2 to 20 mg/kg in total volumes of 0.5 to 1.35 ml.

B) Intravenous injection - female Sprague-Dawley rats (body weight=250 g) were implanted with jugular cannulae by Kelly Drew, to whom I am most grateful. Intravenous

injection of compound 48/80 was performed with doses of 0.5 and 1.0 mg/kg in total volumes of 0.2 to 0.25 ml saline (acute administration) or 0.3 to 0.34 mg/kg in total volumes of 1.25 ml saline (subacute administration).

Following 48/80 administration and observation periods, the animals were sacrificed and the brains processed, fixed and stained as described (Methods section I-i,iia). Tongue was always coprocessed with brain tissue. MCs in both tissues were examined visually for evidence of degranulation

VIII) Statistics and treatment of data

A) Treatment of A-P MC data for statistical analysis - due to slight warpage of some brains during processing, the left and right sides were occasionally cut unsymetrically. Because analysis of variance (ANVAR) requires a blocked (complete) data set, some empty data cells had to be filled prior to analysis. Most statistical packages, including PROPHET, will fill in empty cells according to the mean of the replicates of a particular factor and level. In situations where there is a large variability in the data (as in MC numbers), such a procedure can lead to erroneous or misleading data results. In order to prevent such an occurrence, we developed a method for the estimation of the number of MCs at a particular coordinate of an animal's thalamus based on that animal's total thalamic MC numbers.

The highly precise A-P distribution of rat brain MCs (see above, Fig 15) suggested that, regardless of their overall

numbers, the percentage of total thalamic MCs found at a particular coordinate might be the same for all brains. This was observed, as total MC numbers from each individual section (+0.6 to -3.2 mm) were significantly correlated (at least $P<0.05$) with total thalamic MC numbers across brains (Table 3; for example, see Fig 10). The levels of significance of these correlations were the greatest for those sections whose coordinates were from -0.4 mm to -3.0 mm, as this area contained the greatest MC numbers (Fig 15,16). Thus, the number of MCs at a particular coordinate in thalamus can be reliably estimated by the determination of the total MC numbers of that brain and multiplying that value by the slope of the appropriate correlation.

In order to prevent perturbation of any side differences in MC numbers, the estimated value for the number of MCs per coordinate was multiplied by the percentage of total MCs on the left and right sides of that brain, and these estimated values for left and right MC numbers were entered into data structures. Out of a total of 486 data cells for each sex, 132 were filled for females (27.2%) and 28 were filled for males (5.8%). This procedure for filling data was used only for analysis of MC numbers in study 1 (Results section III-B); the data for age-matched animals (study 2)(Results section III-C) had fewer empty cells and did not require such treatment. The resulting arrays were only used for ANVAR analysis. Filled data were not used for calculation of total

Table 3. Correlations in male and female rats of thalamic MC numbers at a given coordinate with total thalamic MC numbers.

Coord	Males				Females			
(mm)	n	r	Slope	(P<)	n	r	Slope	(P<)
+0.4	6	0.938	0.00989	0.0005	6	0.978	0.01333	0.0001
+0.2	9	0.818	0.00480	0.0025	9	0.973	0.02046	0.0001
0.0	9	0.934	0.00926	0.0005	8	0.989	0.03789	0.0001
-0.2	7	0.922	0.01327	0.0005	9	0.984	0.03283	0.0001
-0.4	8	0.953	0.02592	0.0001	6	0.992	0.04468	0.0001
-0.6	9	0.959	0.03593	0.0001	6	0.871	0.07179	0.01
-0.8	9	0.983	0.05191	0.0001	8	0.987	0.05081	0.0001
-1.0	9	0.914	0.07268	0.0005	6	0.975	0.07593	0.0001
-1.2	8	0.976	0.07641	0.0001	7	0.973	0.08023	0.0001
-1.4	9	0.988	0.09234	0.0001	5	0.997	0.07184	0.0001
-1.6	8	0.948	0.09610	0.0005	7	0.909	0.08814	0.001
-1.8	9	0.976	0.09128	0.0001	6	0.940	0.08616	0.001
-2.0	9	0.968	0.09718	0.0001	8	0.984	0.11343	0.0001
-2.2	8	0.996	0.08121	0.0001	7	0.994	0.07935	0.0001
-2.4	9	0.962	0.06232	0.0001	6	0.996	0.0608	0.0001
-2.6	8	0.979	0.07032	0.0001	7	0.856	0.05286	0.005
-2.8	8	0.894	0.03782	0.0025	9	0.802	0.03934	0.005
-3.0	9	0.932	0.03744	0.0001	5	0.904	0.02937	0.01
-3.2	9	0.946	0.02613	0.0001				

Cryostat sections (10 microns) were taken every 0.2 mm from the anterior (+1.0 mm) to the posterior (-3.6 mm) thalamic borders, stained for MCs and counted as described. Correlations were forced through zero.

MC numbers (Table 5) or for graphical presentation of the A-P distribution of rat brain MCs (Fig 15).

B) Treatment of thalamic nuclear MC data for statistical analysis - empty data cells in the arrays used for the ANVAR analysis of the nuclear distribution of thalamic MCs were filled in a manner analogous to that described above.

Body weights, tissue weights, HA levels and MC numbers were all normally distributed for both sexes. All comparisons, correlations and analyses of variance (ANVARs) were performed with parametric statistics on the PROPHET computer system. Differences between the observed MC numbers of thalamic nuclei were analyzed with the Neuman-Keuls multiple range test. The Scheffe multiple range test was used to determine sex and side differences within coordinates and thalamic nuclei.

I) Histological characterization of rat brain MCs

A) Toluidine blue - brain MCs were metachromatic against a light to moderate orthochromatic background after staining with 0.25% aqueous toluidine blue pH 3.3 (Fig 11). The cells were round to elongated, 9-15 um in diameter, and identical in shape and staining to tongue MCs, which were 10-25 um in diameter (Fig 12). Staining with acidified toluidine blue pH 2.3 (Fig 13, top left) showed these cells with metachromatic granules against an almost indiscernible background. At this pH, the staining of brain MCs was more variable than at the higher pH (Fig 11), with some cells staining lighter than others. For this reason, and the longer staining periods required (see Methods), routine staining of brain MCs for quantification was carried using the 0.25% aqueous toluidine blue pH 3.3 procedure. Most of the toluidine blue positive cells (identified as MCs) were found near vascular channels.

B) Astrablau - brain MCs, stained with ethanolic toluidine blue, appeared identical to those observed after aqueous toluidine blue, but with a stronger orthochromatic background and some staining of neuronal cell bodies (Fig 13, bottom left). Destaining these sections with acidified ethanol and subsequent restaining with Astrablau resulted in a pattern of stained granular cells identical to that observed with toluidine blue, but with almost no background staining (Fig 13, bottom right). Several sections treated with ethanolic toluidine blue had the same MC numbers after

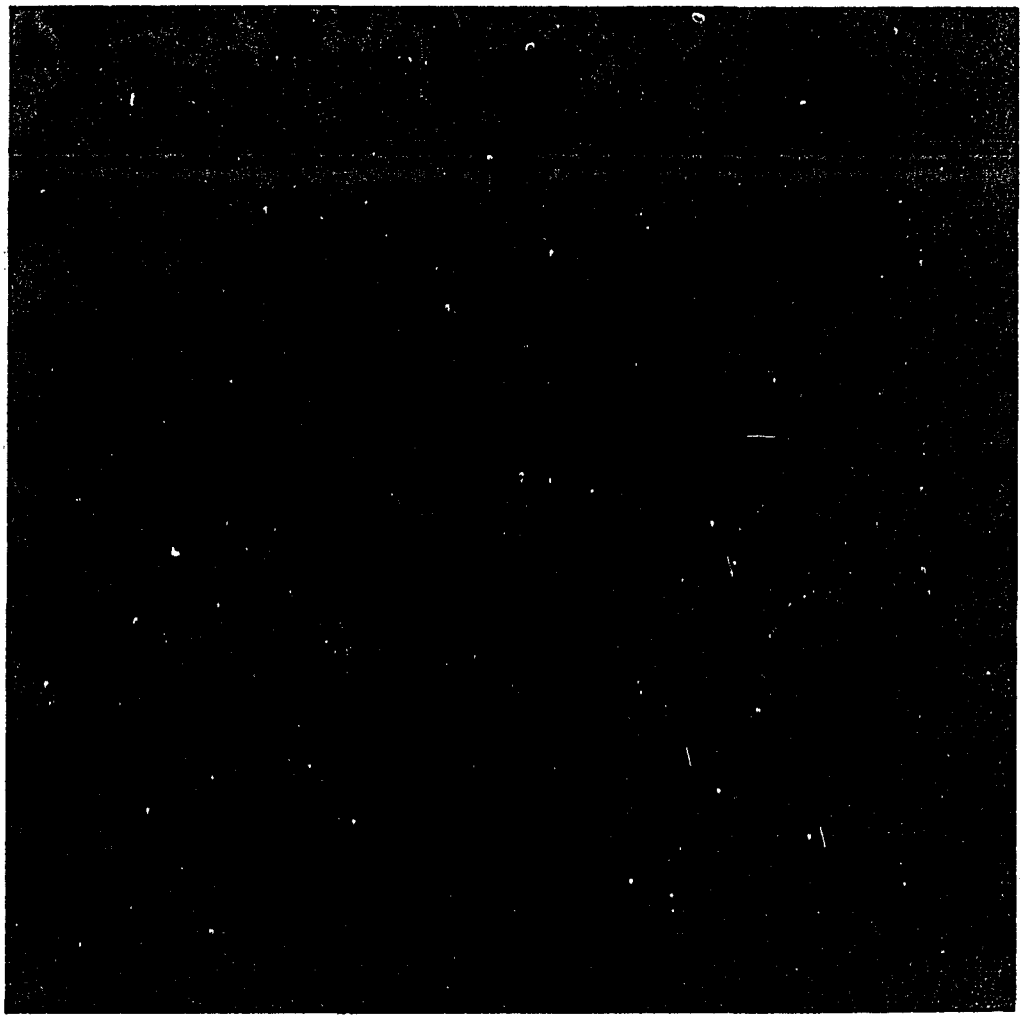

Fig 11. MCs in the paraventricular nucleus of rat thalamus. Sections were fixed with EFA-1% glutaraldehyde and stained with aqueous 0.25% toluidine blue, pH 3.3 (magnification x250).

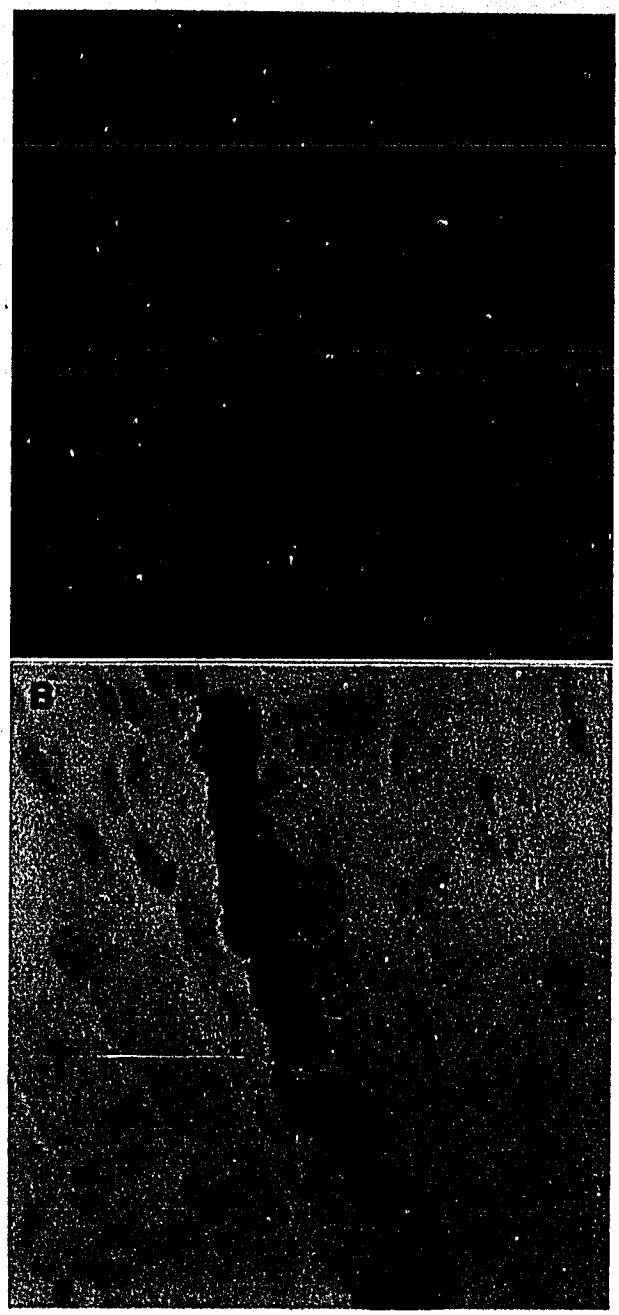

Fig 12. Comparison of rat MCs in brain and tongue. Top: MCs in the medial dorsal nucleus of rat thalamus (diameter=15 um, x250). Bottom: Rat tongue MCs (diamter=20-25 um, x250). Sections were fixed with EFA-1% glutaraldehyde and stained with aqueous 0.25% toluidine blue, pH 3.3.

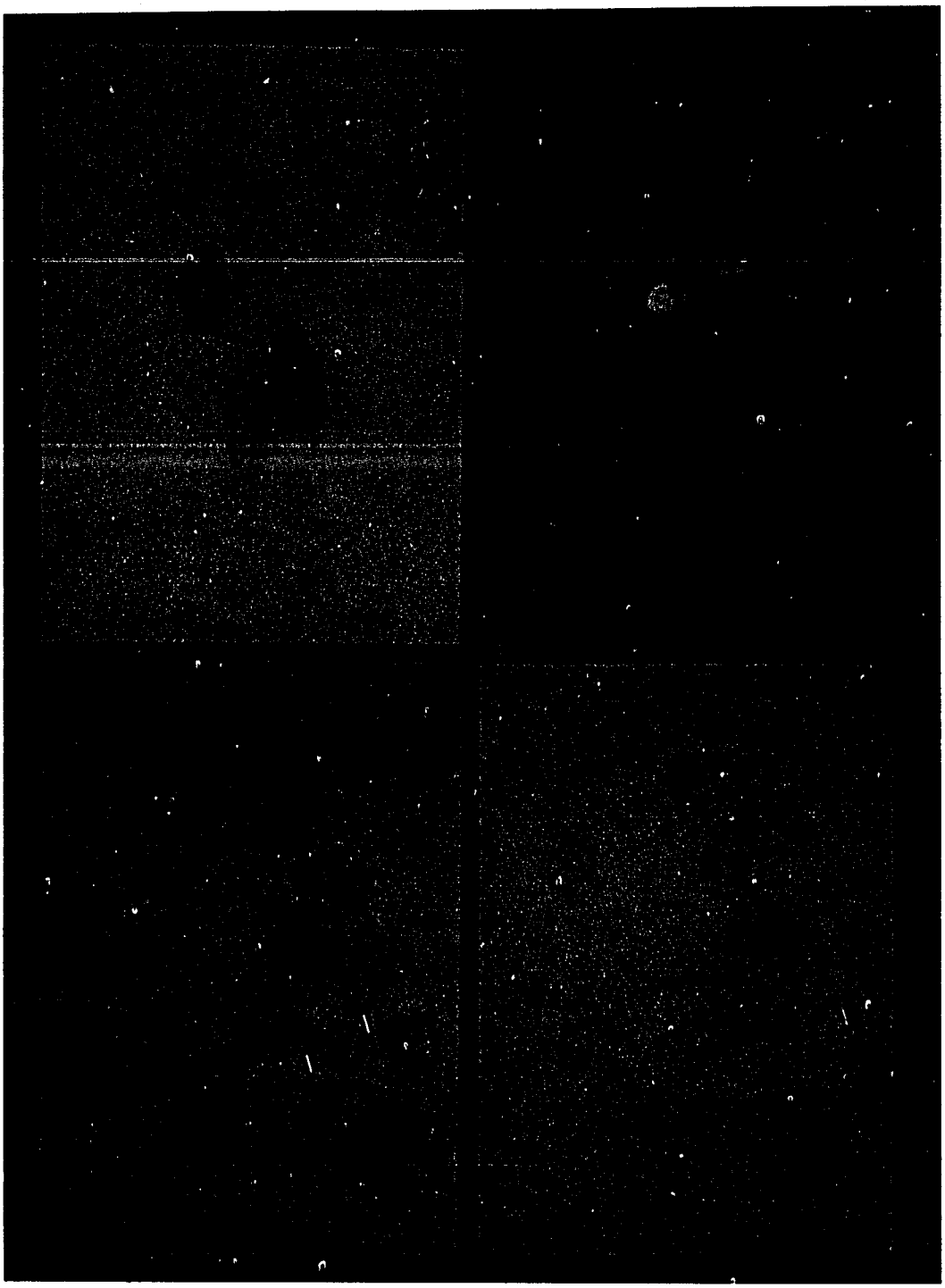

Fig 13. Mast cells in the medial dorsal nucleus of rat thalamus. Upper left: sections were fixed with EFA-1% glutaraldehyde and stained with aqueous 0.25% toluidine blue, pH 2.3 (x400). Bottom: to visualize the same cells with different stains, unfixed sections were stained with 0.005% toluidine blue in 50% ethanol (Lower left), destained, and restained with 0.05% Astrablau FM in 50% ethanol, pH 1 (Lower right, x250). Upper right: histamine in brain MCs was visualized by exposure of dessicated, unfixed cryostat sections to gaseous OPT (x400).

destaining, EFA fixation, and restaining with aqueous toluidine blue pH 3.3.

C) OPT - thalamic sections contained cells that exhibited fluorescent granules when reacted with OPT (Fig 13, top right), suggesting the presence of histamine. The size and shape of these cells were similar to that of thalamic MCs identified with toluidine blue. Control sections, i.e., tissue treated either with OPT but without steam hydration, or treated without OPT but with steam, showed no fluorescence. No OPT-fluorescent cells were found in cortex, basal ganglia, hypothalamus, cerebellum, or brain stem. As with the toluidine blue positive MCs, the OPT fluorescent cells were found near vasculature.

A direct comparison was made of the OPT-positive and toluidine-blue positive cells in thalamus (Fig 14). Of 81 cells observed by either method, 80 percent reacted positively with both treatments, 5 percent reacted only with OPT, and 15 percent reacted only with toluidine blue (Table 4). To ensure that OPT and acetone treatment did not change MC numbers observed with toluidine blue staining, pairs of adjacent cryostat sections (n=18) from anterior thalamus (+0.2 mm) to posterior thalamus (-3.6 mm) were processed separately; the first section of each pair was fixed in EFA and stained with aqueous toluidine blue (pH 3.3), and the second section was treated with acetone/OPT and then stained with ethanolic toluidine blue. MC numbers were not different

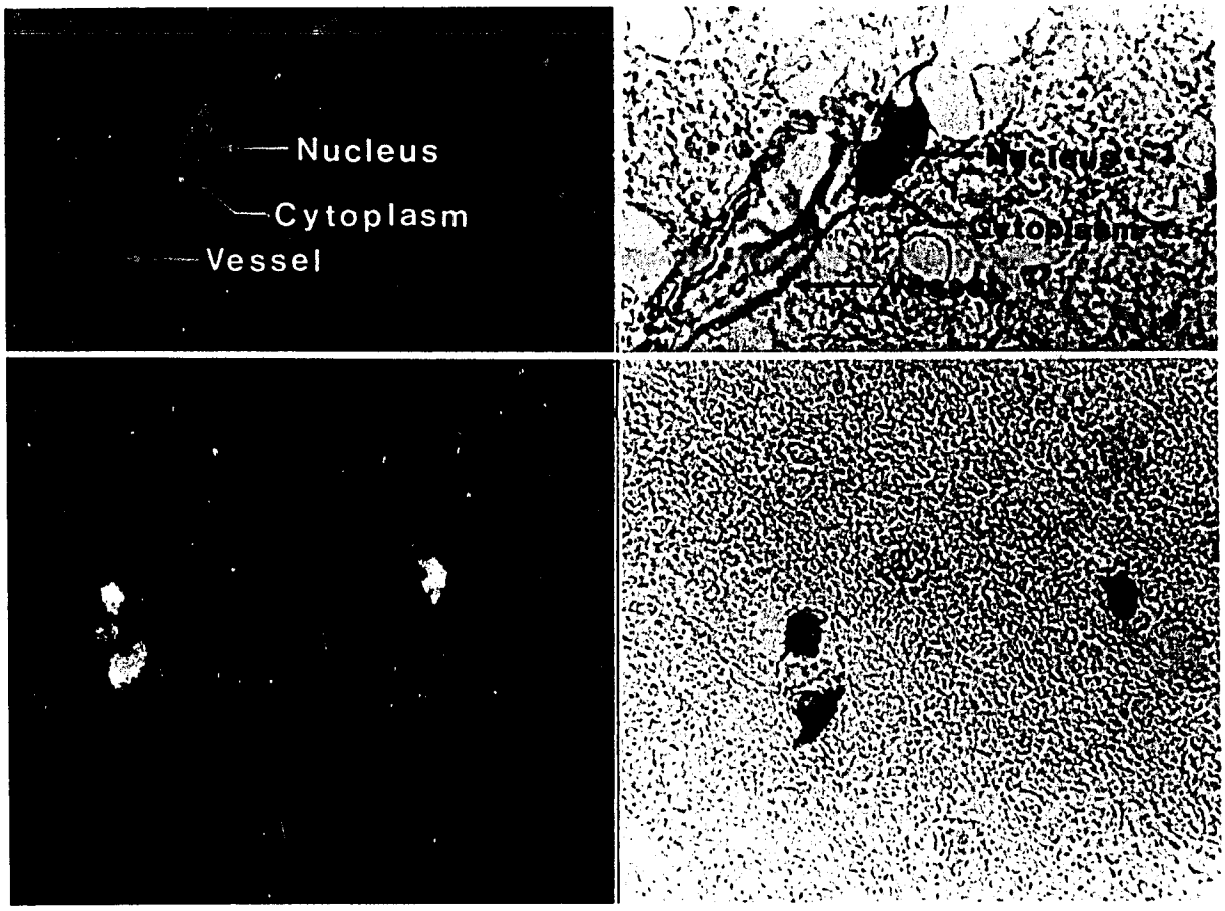

Fig 14. Comparison of OPT-fluorescent cells and toluidine blue staining cells. Lyophilyzed cryostat sections were exposed to gasous OPT (Methods section I-C), photographed under UV light (upper left, x400; lower left, x250) and then rephotographed with visible light following counterstaining with 0.005% toluidine blue in 50% ethanol (upper right, x400; lower right, x250)(Methods section I-C).

Table 4. Rat thalamic MCs visualized by o-phthaldahyde (OPT) and toluidine blue (TB).

	OPT+/TB+	OPT+/TB-	OPT-/TB+	SUM
Number of cells:	65	4	12	81
Fraction of total:	80%	5%	15%	100%

Fresh cryostat sections were exposed to OPT vapor as described. Fields of fluorescent suspected MCs were photographed under blue light (x400), fixed and stained under the cover slip with ethanolic toluidine blue, and re-photographed in bright field (x400). Cells were classified as being positive for both OPT and toluidine blue (OPT+/TB+), positive only to OPT (OPT+/TB-), or positive only for toluidine blue (OPT-TB+).

between the two methods, and both methods exhibited similar MC distributions (not shown).

D) Paraformaldehyde - tissue sections treated with formaldehyde contained fluorescent cells. These cells were found only in thalamus, were situated in or near vasculature and had similar size and shape characteristics as toluidine blue-staining cells. Counterstaining paraformaldehyde-treated sections with aqueous toluidine blue resulted in massive rippling of the sections. However, careful focussing indicated that there was a one to one correspondence between fluorescent cells and toluidine blue-staining MCs. Control sections (treated as experimental sections but without paraformaldehyde) also contained cells that showed considerable fluorescence and these cells had characteristics similar to those found in formaldehyde-treated sections. There was also a one to one correspondence between these autofluorescent cells and toluidine blue-staining MCs. Thus, no inferences pertaining to the 5HT content of rat brain MCs can be made. As tongue tissue could not withstand the present experimental procedure, control sections could not be run.

II) Pharmacological characterization of rat MCs with compound 48/80

In order to ascertain the ability of rat brain MCs to degranulate in response to peripheral administration of the releasing agent compound 48/80 (Paton, 1957), 48/80 was

injected into female rats both intraperitoneally and intravenously.

A) Peritoneal administration - a partial dose-response curve was obtained by injecting female rats (body weight=250 g) with compound 48/80 in normal saline at 2,5,10 and 20 mg/kg (n=1 for each dose) in a total volume of 0.5 to 1.35 ml. Following a 2 mg/kg dose, the animal became prostrated and was hyperventilating within 5 min, and exhibited signs of severe systemic shock within 10 min. Paws, ears and nose became extremely swollen and red. After 25 min, the symptoms became less severe and the animal was recovered by 45 min. At this time, the animal was sacrificed.

All doses above 2 mg/kg resulted in similar symptoms, with prostration occurring within 5 min, followed by coma and death (at 35 min after 5 mg/kg and 12 min after 20 mg/kg). Following death, animals were processed as described.

Whereas 48/80 was effective in causing the release of HA from peripheral MCs, as evidenced by the systemic shock and the partial (2 mg/kg) to moderate (20 mg/kg) degranulation observed in tongue MCs (not shown), these doses failed to elicit any degranulation of brain MCs.

B) Intravenous administration

i) Acute administration - an attempt was made to determine the LD_{50} of 48/80 following intravenous injection. Doses of 0.5 and 1.0 mg/kg in volumes of 0.2-0.25 ml were administered into jugular cannulas and the animals observed. Following 0.5 mg/kg, the symptoms and time course were

similar to that of peritoneal injection of 5 mg/kg 48/80. Similarly, the results following jugular administration of 1.0 mg/kg were almost identical to that following intra-peritoneal injection of 20 mg/kg. Due to the paucity of cannulated animals available, no attempt was made to extend the dose-response curve to lower doses. Acute jugular injection was successful in degranulating peripheral MCs but was completely ineffective on brain MCs.

ii) Prolonged administration - in an attempt to determine whether a time factor rather than a dose factor was responsible for the inability of 48/80 to affect brain MCs, female rats were given multiple, sublethal doses starting at 0.25 mg/kg, each dose being administered when the animal seemed to be recovering from the previous dose. Two animals were given a total of 0.3 and 0.34 mg/kg in a total volume of 1.25 and 0.93 ml saline respectively over a 2 hr period. Whereas this regimen caused severe discomfort in one animal and death in the other, the results were as before, namely that tongue MCs became extensively degranulated and brain MCs remained unaffected.

Thus, in none of the administration protocols attempted here did 48/80 have any histologically verifiable effect on brain MCs.

III) Quantification of brain MCs

A) Anatomical distribution - rat brain MCs were highly localized to the thalamus. In four whole brains (+6.0 to

-8.0 mm, brain stem and part of cerebellum excluded), more than 98 percent of the MCs were in thalamus (+0.8 to -3.6 mm), with the remaining 2 percent in parietal cortex or, more rarely, near the optic chiasm. In 20 brains examined from anterior commissure to rostral midbrain (+2.0 to -3.6 mm, inclusive of the thalamus), nearly all MCs were within thalamus, and none were found in hypothalamus or basal ganglia.

Most of the MCs observed were situated in or near vascular elements. Some cells, however, showed no association with blood vessels.

B) MC numbers in adult male and female rats (study 1)

i) Thalamic MC numbers and anterior-posterior (A-P) thalamic MC distribution - the thalami of adult rats were examined for brain MCs as described. Brain MC numbers varied greatly among animals. Values for males ranged from 1,490 to 19,103 cells per brain (mean$\pm$SEM: 6,966$\pm$2,217), while values for females ranged from 1,200 to 31,950 cells per brain (7,854$\pm$3,231) (Table 5). Despite this variability, all brains exhibited a striking and reproducible MC distribution within the thalamus along the anterior-posterior plane (Fig 15). Peak MC numbers were observed at the level of the lateral habenular nucleus (-1.7 mm) and declined to zero at the anterior (+0.6 mm) and posterior (-3.6 mm) borders of the thalamus (Fig 15).

ii) MC numbers as a function of sex and side of brain - a three-way analysis of variance of the filled data for A-P

Table 5. Summary of thalamic MC numbers, ages and body weights from all studies.

	Males	Females
Distributional Study 1		
MC numbers: mean±SEM	6966±2217	7854±3231
CV%, n	95%, 9	123%, 9
range	1490-19130	1200-31950
Body weight: mean±SEM	329±9 g	276±10 g
CV%, range	8%, 295-367 g	10%, 250-315 g
Age (estimated)		
mean±SEM	10.5±0.3 wks	> 16 wks
range	9.3-11.8 wks	15 - > 16 wks
Distributional Study 2 (Age match)		
MC numbers: mean±SEM	6318±1156	6825±1969
CV%, n	52%, 8	82%, 8
range	696-11532	210-18190
Body weight: mean±SEM	342±7 g	228±6 g
CV%, range	6%, 307-375 g	7%, 205-255 g
Age (actual):		
mean±SEM	12.1±0.1 wks	12.4±0.1 wks
CV%, range	2%, 11.9-12.6 wks	4%, 11.9-12.9 wks
Age (estimated):		
mean±SEM	11.1±0.2 wks	12.8±0.6 wks
range	9.6-11.5 wks	10.3-16 wks
Biochemical analysis of HA		
MC numbers*: mean±SEM	11626±1561	7944±1164
CV%, n	63%, 22	72%, 24
range	1094-30535	815-19624
Body weight: mean±SEM	297±8 g	254±3 g
CV%, range	12%, 230-365 g	6%, 225-285 g
Age (estimated)		
mean±SEM	9.5±0.3 wks	approx 16 wks
range	8.4-11.7 wks	12.5 - > 16 wks

MC numbers (studies 1 and 2) were obtained by counting coronal diencephalic sections and multiplying the sum of the observed MC numbers across thalamus by 20 to account for the volume of thalamic tissue measured (Methods section II). Estimated ages were obtained by fitting individual animal weights to an age-weight chart (Fig 28).

*Estimated total MC numbers (biochemical analysis of HA) were obtained from the MC number at a single coordinate divided by the slope of the appropriate correlation (Methods section III-B) and multiplying that value by 20.

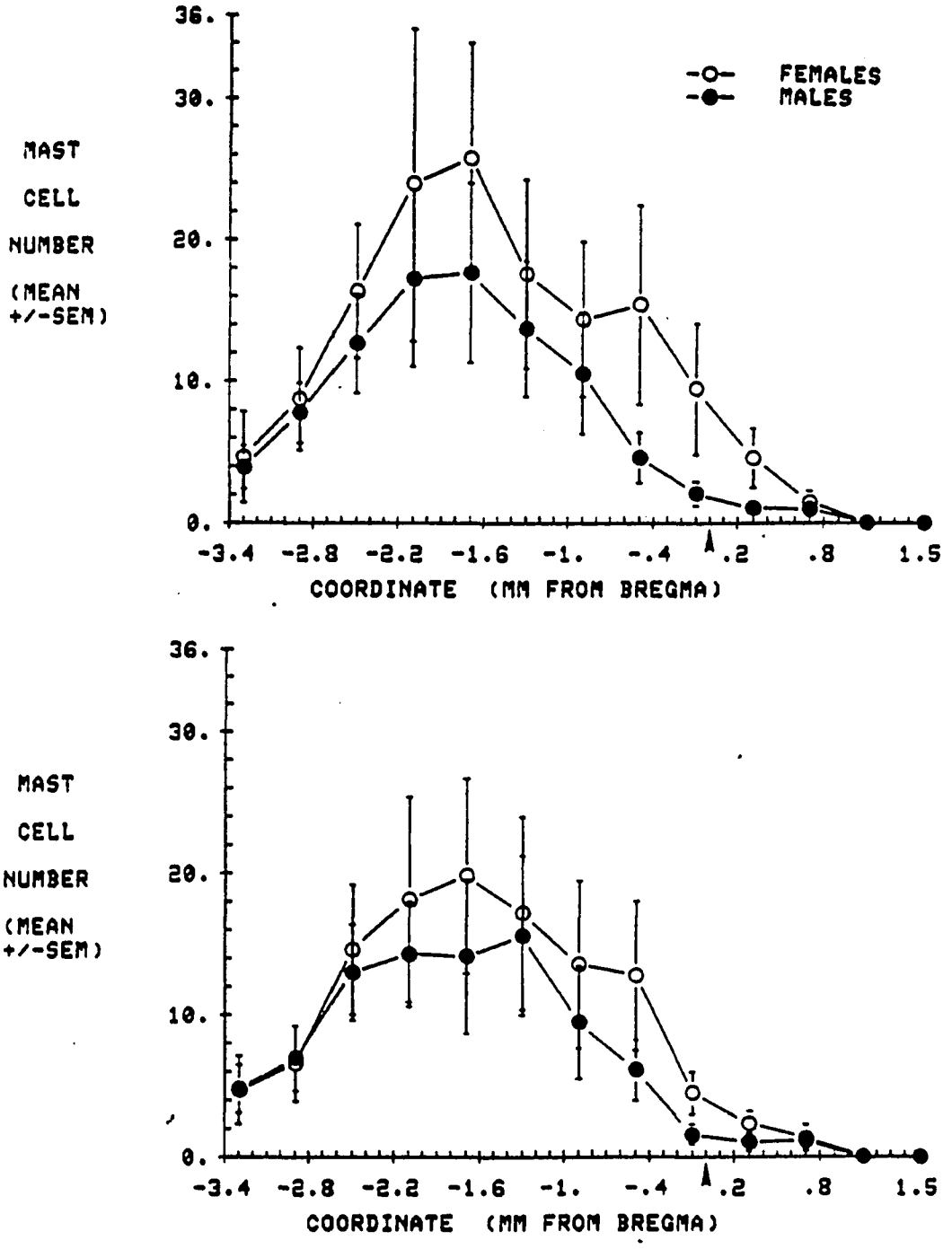

Fig 15. Anterior-posterior distribution of MC numbers in rat thalamus - study 1. Fixed coronal cryostat sections were stained with 0.25% aqueous toluidine blue (pH 3.3) and examined for mast cells. Mean MC numbers (± S.E.M.; n=9) of left (top) and right (bottom) sides of brain are shown. No cells were observed posterior to -3.6 mm. Abscissa shows the distance (mm) from bregma (0, arrow), positive values being anterior. Analysis of variance of these data showed significant effects of coordinate ($P<0.0005$), sex ($P<0.025$) and side of brain ($P<0.0005$).

MC numbers showed significant effects of coordinate ($P<0.0005$), sex ($P<0.025$), and side of brain ($P<0.0005$) on MC numbers. Interactions among these factors were not statistically significant. Mean MC numbers were higher for females than for males on both sides of brain (Fig 15), but no single coordinate values were significantly different from any other coordinate by multiple comparison analysis, indicating that the sex difference is distributed over the entire thalamus.

The left side of the brain had significantly more MCs than the right side. As with the sex difference, there were no significant side differences at any single coordinate, indicating that the side difference is also distributed over the entire thalamus. Although there was no significant sex-side interaction in the analysis of variance, left-right differences in MCs appeared to be more prominent in females (Fig 15). Eight out of nine female brains had greater MC numbers in the left hemisphere ($X^2=5.4$; $P<0.025$). In males, individual brains exhibited either left or right asymmetry with about the same frequency.

iii) Thalamic nuclear distribution of rat brain MCs - rat brain MCs were unevenly distributed among thalamic nuclei (Table 6). Six of the 32 thalamic nuclei identified contained 70-75% of thalamic MCs, and their rank order was approximately the same for both sexes and sides of brain. A three-way analysis of variance of these data showed significant effects of nucleus ($P<0.0005$), sex ($P<0.05$), and

Table 6. Nuclear distribution of MCs in rat thalamus.

	FEMALE		MALE	
NUCLEUS	LEFT	RIGHT	LEFT	RIGHT
Ventral[a]	54±19	52±15	48±15	51±15
Med. Dorsal[b]	33±12	30±12	29±10	30±13
Vent. Dorsal[b]	26±9	21±10	25±9	21±5
Lateral	21±9	18±6	19±6	14±4
Paraventric.	21±8	17±7	14±5	12±5
Reuniens	21±14	12±6	6±2	6±3
Posterior	12±3	9±3	7±2	11±3
Lat. Gen.	10±4	8±3	9±3	9±3
Parafasc.	5±3	5±2	3±1	4±1
Ant. Med.	7±4	2±1	1±0	0

Fixed coronal diencephalic cryostat sections were stained with 0.25% aqueous toluidine blue (pH 3.3) and examined for MCs. Shown are the number of MCs (mean+S.E.M.) counted in particular nuclei. Total numbers of MCs can be estimated by multiplying by 20 (see Methods).

[a] Total MC numbers (i.e. left + right) for this nucleus significantly different ($P<0.01$) from all other nuclei.

[b] Total MCs in these nuclei significantly different ($P<0.01$) from ventral nucleus and all other nuclei.

side of brain ($P<0.005$), with no significant interactions. MC numbers in ventral, medial dorsal, and ventral dorsal nuclei were significantly greater ($P<0.01$) than all other nuclei in both sexes (Table 6). No individual nucleus exhibited side or sex differences by multiple comparison analysis, indicating that these differences are spread over all nuclei.

C) Thalamic MC numbers in adult male and female (matched for age, study 2) - the significant sex and side differences in brain MC numbers observed in rats of similar weights (section III-B-ii) prompted an investigation as to whether similar results might be obtained in rats of similar ages. Although the exact ages of the rats used in the previous study were unknown, male and female adult rats of similar ages (12 weeks, Table 5) were processed for brain MC quantification as described. Only A-P data were collected. As found previously, rat brain MCs exhibited a striking A-P distribution (Fig 16). Female rats had mean thalamic MC numbers higher than males but were not different by group t-test. Both sexes displayed marked variability in brain MC numbers between individuals (Table 5). In contrast to the previous results, ANVAR indicated that there were no differences in MC numbers either between sides of brain ($P>0.5$) or sexes ($P>0.5$).

To examine which factor might have been the cause of the difference in findings between the present data (age-matched, study 2) and the data presented above (study 1), data from

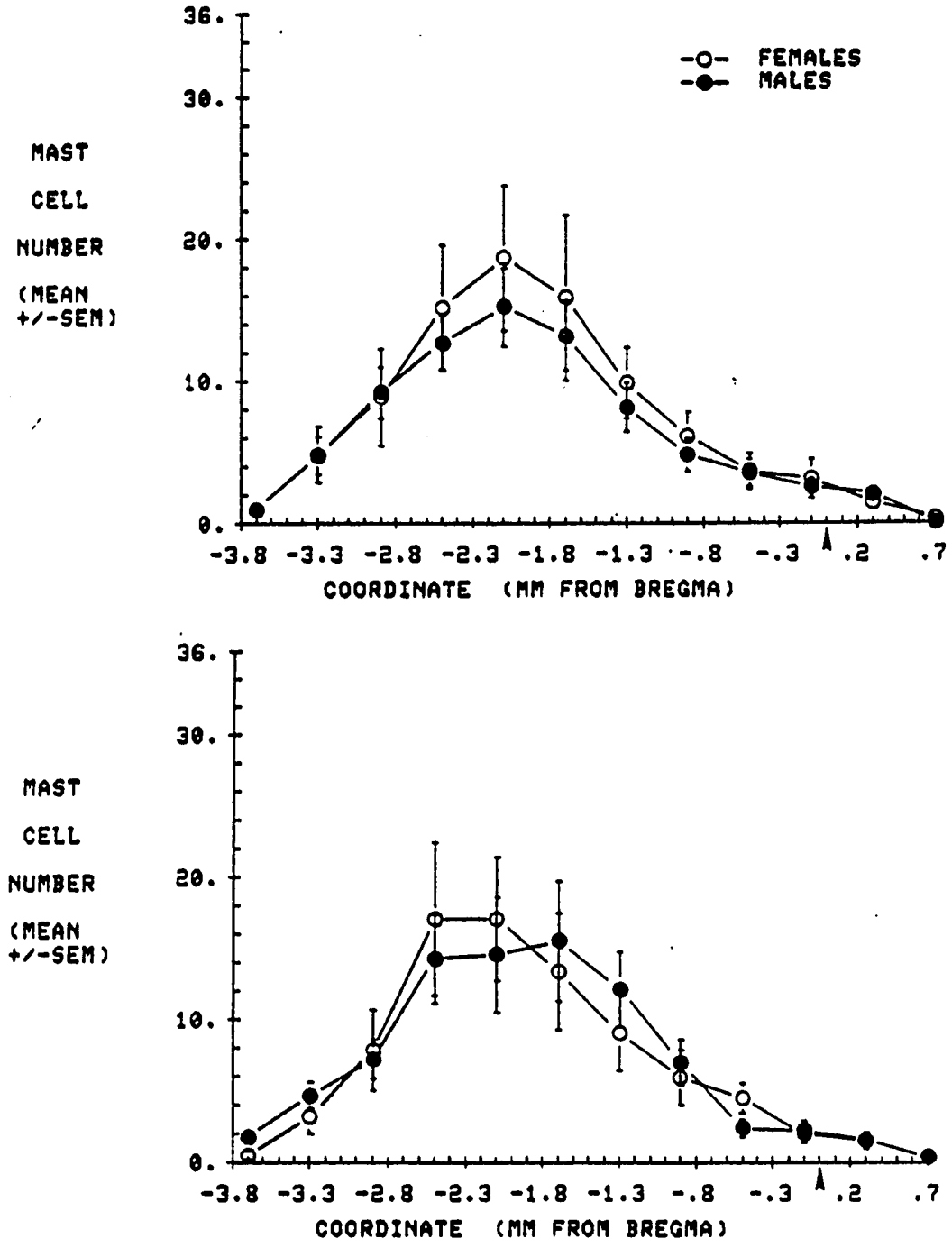

Fig 16. Anterior-posterior distribution of MC numbers in rat thalamus - study 2 (age-matched animals). Fixed coronal cryostat sections were stained with 0.25% aqueous toluidine blue (pH 3.3) and examined for mast cells. Mean MC numbers (± S.E.M.; n=8) of left (top) and right (bottom) sides of brain are shown. No cells were observed posterior to -3.6 mm. Abscissa shows the distance (mm) from bregma (0, arrow), positive values being anterior. Analysis of variance of these data showed significant effects of coordinate ($P<0.0005$), but no significant effects of sex or side of brain.

the two experiments were combined in several ways and analyzed by ANVAR. Such analysis (2-way ANVAR: level 1=experiment, level 2=sex) indicated that there were no differences in total MC numbers between the males of studies 1 and 2 or between the females of studies 1 and 2 ($P>0.5$). Analysis of MC numbers by side (rather than total numbers)(2-way ANVARs, four sets of data: level 1=coordinate, level 2=experiment) indicated no differences between the two studies in MC numbers in the left sides of males ($P>0.1$), in the right sides of males ($P>0.3$) or in the right sides of females ($P>0.05$). However, ANVAR of the data from the left side of females of studies 1 and 2 indicated that there was a significant ($P<0.02$) difference in MC numbers on this side of females between the studies. ANVAR of the combined data from the two studies (one large data set with a total n of 17; 3-way ANVAR: level 1=coordinate, level 2=sex, level 3=side-of-brain) indicated that the superset of MC values retained significant sex ($P<0.02$) and side of brain ($P<0.005$) differences in MC numbers.

IV) Correlation of MC numbers and HA values from individual rat brains

Just as correlations of the MCs per coordinate vs total MC numbers can be used to estimate the number of MCs at a particular coordinate, so might the number of MCs at a coordinate be used to estimate the total number of MCs in a particular brain. Therefore, this investigator developed a

method to remove and count a single section from the middle of thalamus, allowing the remainder of the thalamus to be homogenized and assayed for HA and its metabolite tMH (Methods section III).

Correlations of the number of MCs at a particular coordinate on one side of brain with the total number of MCs on that side proved to be significant across a wide range of coordinates (males, Table 7; females, Table 8). Because the A-P coordinates on the left and right sides of the slice used for MC estimation were not always identical (but were never more than 0.2 mm apart), total MC numbers were estimated for the two sides separately.

A) Estimated thalamic MC numbers - mean thalamic MC numbers estimated for females were similar to those found previously (7944±1164, Table 5). Male values, however, were considerably higher than those seen before (11,626±1561, Table 5). There was no difference in the estimated MC numbers between the sexes by group t-test. Both sexes exhibited the large variability in numbers that had been seen with direct quantification (Table 5). Similar to previous data, females had a larger variation in their MC numbers than did males. There were no differences by group t-test between the present MC numbers and those found in my previous studies (studies 1 and 2).

B) Thalamic HA levels and correlation with thalamic MC numbers - thalamic HA levels were higher and more variable (males: 451±60 ng/g; females: 264±27 ng/g; Table 9) than

Table 7. Correlations of male rat thalamic MC numbers observed at a given coordinate with total thalamic MC numbers observed for that side.

Coordinate		Left Side			Right Side		
(mm)	n	r	Slope	(P<)	r	Slope	(P<)
-0.4	8	0.917	0.02868	0.0005	0.927	0.02247	0.0005
-0.6	9	0.971	0.02514	0.0001	0.943	0.04954	0.0001
-0.8	9	0.957	0.05758	0.0001	0.964	0.04326	0.0001
-1.0	9	0.902	0.06456	0.0005	0.927	0.08109	0.0005
-1.2	8	0.963	0.07009	0.0001	0.953	0.08658	0.0001
-1.4	9	0.986	0.08374	0.0001	0.982	0.10134	0.0001
-1.6	8	0.950	0.09534	0.0001	0.936	0.09609	0.0005
-1.8	9	0.980	0.10291	0.0001	0.967	0.07825	0.0001
-2.0	9	0.989	0.10709	0.0001	0.914	0.08543	0.0005
-2.2	8	0.985	0.09062	0.0001	0.981	0.06939	0.0001
-2.4	9	0.945	0.06043	0.0001	0.951	0.06326	0.0001
-2.6	8	0.994	0.06803	0.0001	0.944	0.07257	0.0005
-2.8	8	0.858	0.03776	0.003	0.911	0.03702	0.001
-3.0	9	0.939	0.03481	0.0001	0.916	0.04028	0.0005

Cryostat sections (10 microns) were taken every 0.2 mm from the anterior (+1.0 mm) to the posterior (-3.6 mm) thalamic borders, stained for MCs and counted as described. Correlations were forced through zero.

Table 8. Correlations of female rat thalamic MC numbers observed at a given coordinate with total thalamic MC numbers observed for that side.

Coordinate		Left Side			Right Side		
(mm)	n	r	Slope	(P<)	r	Slope	(P<)
-0.4	6	0.991	0.05107	0.0001	0.969	0.03593	0.0005
-0.6	6	0.906	0.07569	0.005	0.821	0.06732	0.024
-0.8	8	0.955	0.04482	0.0001	0.992	0.05807	0.0001
-1.0	6	0.995	0.06783	0.0001	0.943	0.08842	0.001
-1.2	7	0.968	0.06792	0.0001	0.975	0.09681	0.0001
-1.4	5	0.995	0.06608	0.0001	0.985	0.07873	0.0005
-1.6	7	0.906	0.09059	0.002	0.912	0.08456	0.002
-1.8	6	0.899	0.07744	0.006	0.969	0.09593	0.0005
-2.0	8	0.985	0.13072	0.0001	0.980	0.09187	0.0001
-2.2	7	0.993	0.07066	0.0001	0.985	0.09066	0.0001
-2.4	6	0.995	0.05392	0.0001	0.984	0.06980	0.0001
-2.6	7	0.795	0.04583	0.018	0.904	0.06115	0.002
-2.8	9	0.815	0.04028	0.004	0.768	0.03793	0.009
-3.0	5	0.879	0.03192	0.021	0.925	0.02597	0.008

Cryostat sections (10 microns) were taken every 0.2 mm from the anterior (+1.0 mm) to the posterior (-3.6 mm) thalamic borders, stained for MCs and counted as described. Correlations were forced through zero.

Table 9. HA content (ng), HA concentrations (ng/g) and tissue weights in male and female rat brain.

Parameter	Sex	Mean	S.E.M.	Range	C.V.(%)
Thalamic Weight (g)	M:	0.0812	0.0024	0.0631-0.1015	13.8
	F:	0.0696*	0.0025	0.0487-0.0930	17.6
Whole Brain Weight (g)	M:	1.945	0.023	1.787-2.260	5.5
	F:	1.866*	0.017	1.667-2.005	4.6
Thalamic Histamine (total ng)	M:	36.2	4.7	6.0-81.3	60.6
	F:	18.6*	2.2	3.6-47.0	57.5
Thalamic Histamine (ng/g)	M:	451.0	59.8	89.2-1137.6	62.2
	F:	264.3*	27.2	59.8-596.4	50.4
Non-thal. Histamine (total ng)	M:	75.3	3.9	48.2-110.4	24.3
	F:	80.5	3.9	53.0-117.4	23.8
Non-thal. Histamine (ng/g)	M:	41.0	2.1	25.5-57.3	24.2
	F:	45.4	2.2	28.1-66.9	23.1
Whole brain Histamine (total ng)	M:	112.4	6.9	66.3-167.9	28.7
	F:	99.1	5.2	69.0-162.7	25.9
Whole brain Histamine (ng/g)	M:	57.8	3.4	33.1-89.2	27.8
	F:	53.7	2.7	37.0-83.7	24.7

Frozen brains from male (n=22) and female (n=24) rats were dissected into thalamic and non-thalamic (i.e., the remainder of the brain) portions and assayed for HA as described. Whole brain weights and whole brain HA amounts (ng) were calculated as the sum of thalamic and non-thalamic values. Whole brain HA concentrations (ng/g) are derived from whole brain weights (g) and whole brain histamine (ng). Shown are the mean, standard error of the mean (S.E.M.), range and coefficient of variation (C.V.).

* Significantly different ($P<0.01$) from the corresponding male value by group t-test.

those found in previous studies (see Table 1) and were significantly different ($P<0.01$) between sexes by group t-test. Indeed, the variation of these values (values ranging over an order of magnitude, Table 9) was of a similar nature to that of the MC numbers (Table 5). Thalamic HA content (ng per thalamus) was significantly correlated with the estimated thalamic MC numbers both in males ($r=0.843$, $P<0.0001$; Fig 17 top) and in females ($r=0.678$, $P<0.0005$; Fig 17 bottom). The slopes of the fitted lines, indicative of the mean HA content per thalamic MC, were 2.52 and 1.27 pg/cell for males and females respectively, significantly different by analysis of covariance (ANCOVAR)($P=0.013$). The Y-axis intercepts, indicative of the mean thalamic HA content outside of MCs, were 6.8 ng in males and 8.5 ng in females.

Thalamic MC numbers were also highly correlated with thalamic HA levels in males ($r=0.809$, $P<0.0001$) and females ($r=0.654$, $P<0.001$)(Fig 18). The Y-intercepts of these lines, indicative of the non-MC HA levels of the thalamus, were 90.5 ng/g and 143 ng/g in males and females, respectively. Again, the HA levels in males and females were different ($P<0.01$) by group t-test.

C) Non-thalamic HA levels and correlation with thalamic MC numbers - non-thalamic HA (i.e., the amount of HA outside of thalamus) was lower in both content and variability than thalamic HA (males: 41±2 ng/g; females: 45±2 ng/g; see Table 9). The correlation of non-thalamic HA content (ng) with thalamic MC numbers was not significant in either sex (Fig

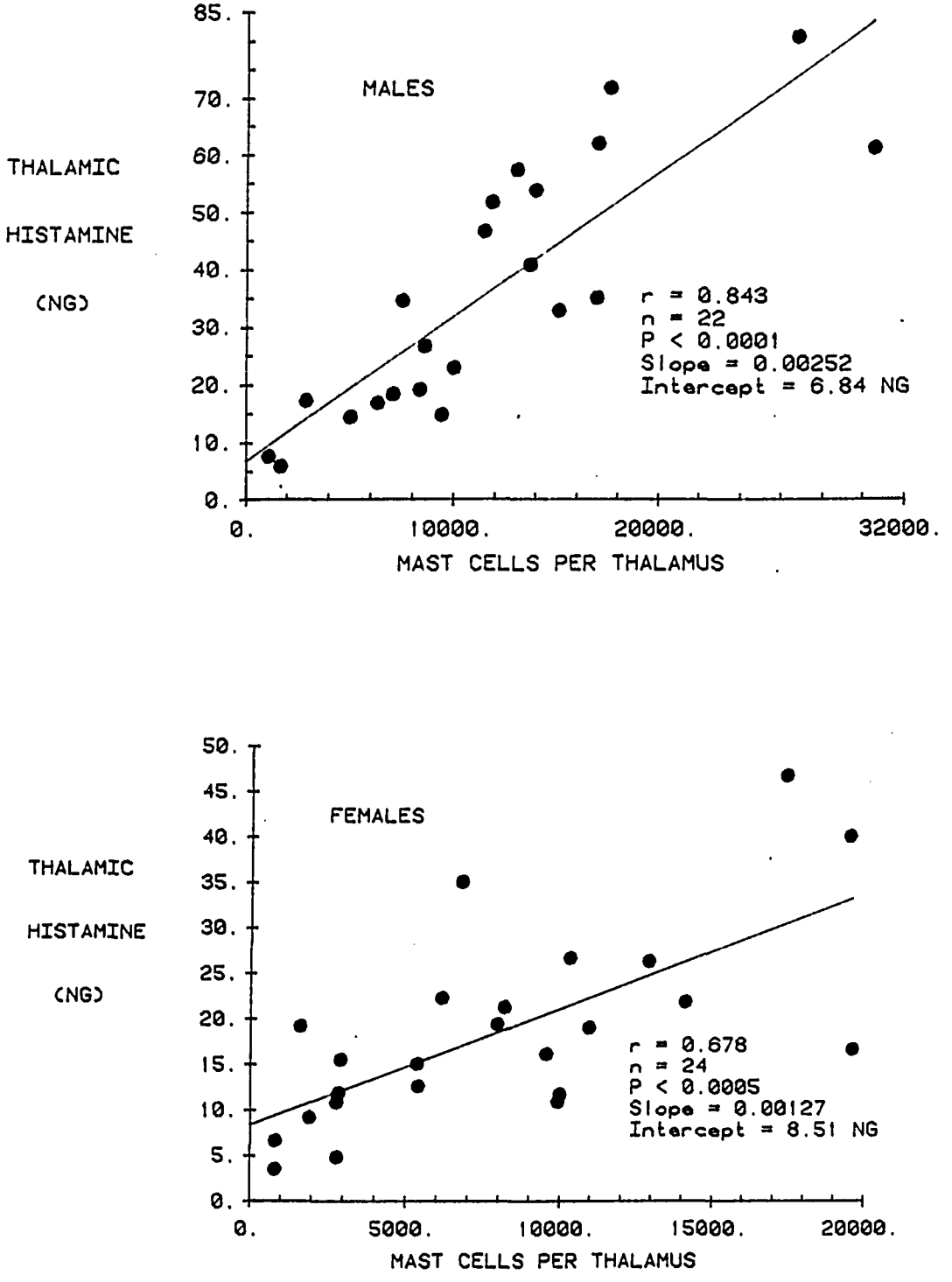

Fig 17. Correlation of estimated thalamic MC numbers with thalamic HA content (ng) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

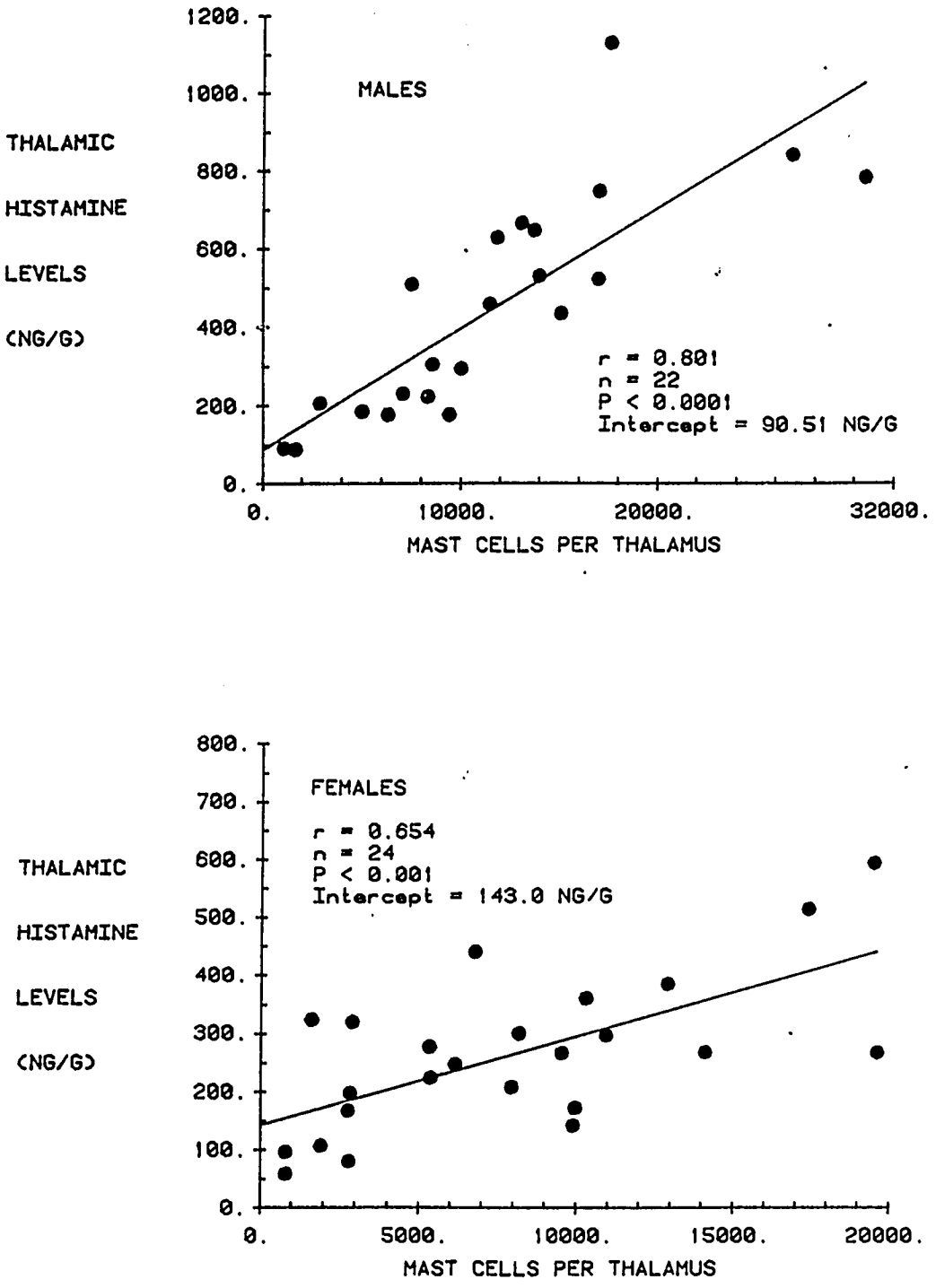

Fig 18. Correlation of estimated thalamic MC numbers with thalamic HA levels (ng/g) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

19). Similar correlations with non-thalamic HA levels (ng/g) were not significant for males ($r=0.122$, $n=22$, $P>0.05$) and border-line significant for females ($r=0.406$, $n=24$, $P=0.049$)(curves not shown). Comparison of the male and female correlations of non-thalamic HA levels with thalamic MC numbers by Fisher's z transformation (Zar, 1974) indicated no differences between the two correlations ($Z=0.663$, $P>0.5$). The common (or weighted) correlation coefficient (r_w) (obtained from the common z value, z_w; Zar, 1974) indicated that the correlation obtained from the combined male and female data was not significant ($z_w=0.271$, $r_w=0.265$, $P>0.05$). There were no significant differences between males and females in either non-thalamic HA amounts or levels.

D) Whole brain HA levels and correlation with thalamic MC numbers - whole brain HA levels (males: 58±3 ng/g; females: 54±3 ng/g; Table 9) resembled those found previously by others. There were no differences between sexes in HA content or levels. Correlations of the whole brain HA content (ng) with thalamic MCs were significant in both sexes (males: $r=0.694$, $n=22$, $P<0.0005$; females: $r=0.576$, $n=24$, $P<0.003$) and indicated a non-MC HA content of 77 ng in males and 79 ng in females (curves not shown). The slopes of these lines from whole brain data indicated a MC HA content of 3.1 pg HA/MC in males and 2.6 pg HA/MC in females.

Whole brain HA levels (ng/g) were also significantly correlated with thalamic MC numbers in both sexes ($P<0.002$ for both sexes, Fig 20). The Y-intercepts indicated that

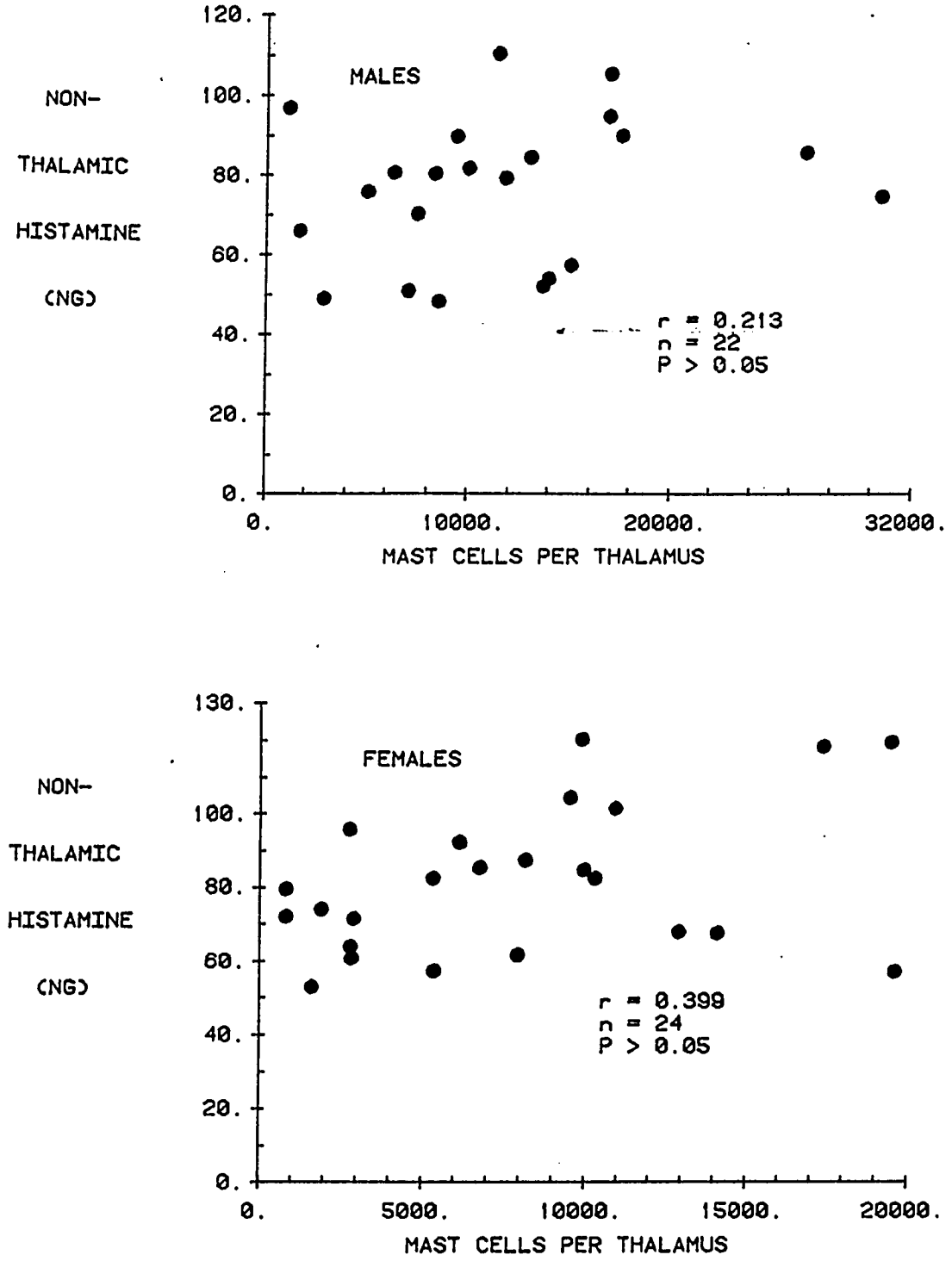

Fig 19. Correlation of estimated thalamic MC numbers with non-thalamic HA content (ng) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

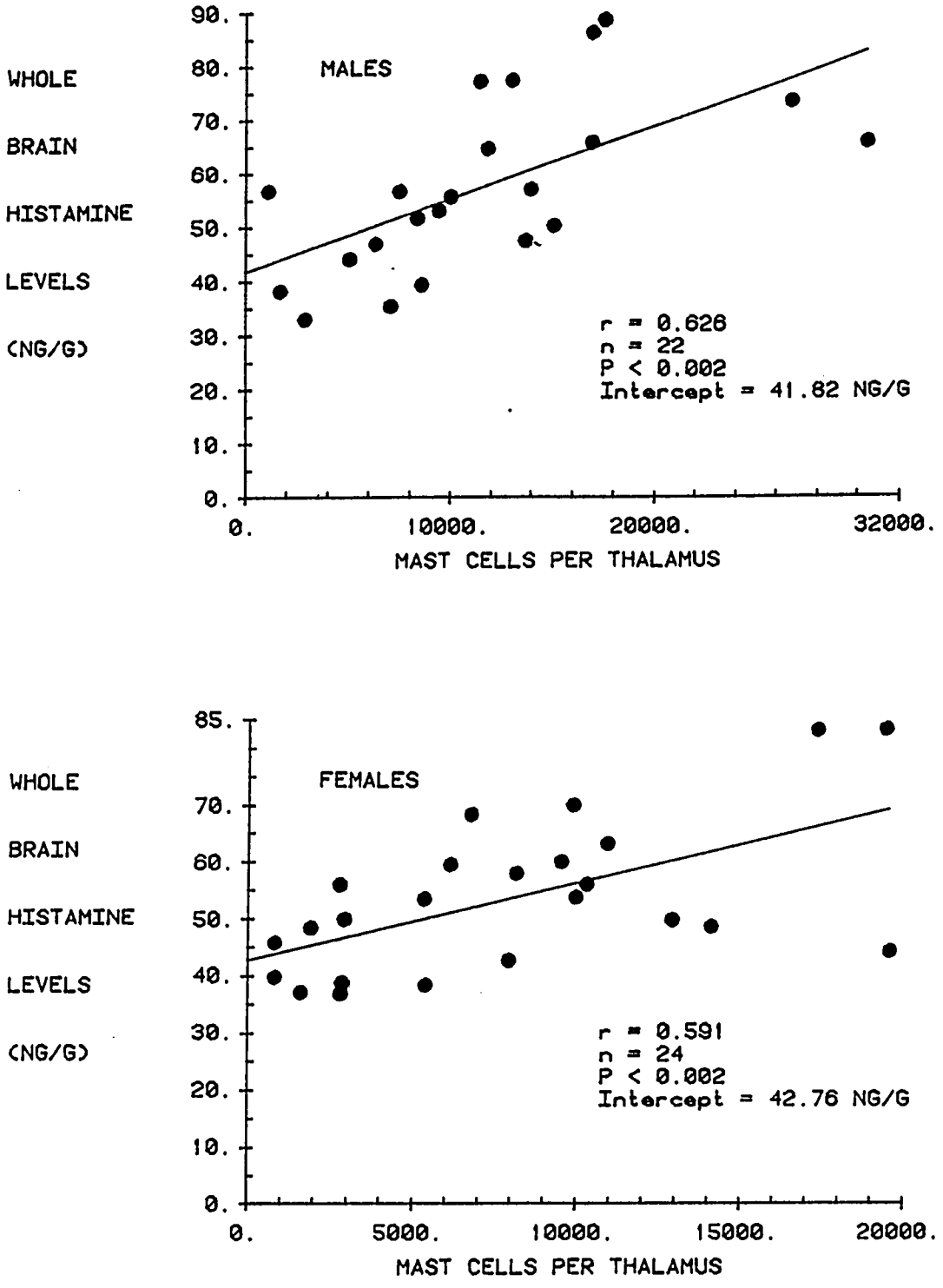

Fig 20. Correlation of estimated thalamic MC numbers with whole brain HA levels (ng/g) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

adult rat brain contained levels of approximately 42 ng/g of non-MC HA.

E) Other correlations - other factors do not seem to account for the observed thalamic HA-MC correlations. Thalamic weights were not correlated with either thalamic MC numbers or with thalamic HA levels (ng/g). This is consistent with our findings that thalamic MC numbers correlated as well with thalamic HA concentrations (ng/g) as they did with thalamic HA content (ng) in both sexes. Body weight was unrelated to thalamic MC numbers or to thalamic HA content (ng) or levels (ng/g). Interestingly, body weight was negatively correlated ($P<0.005$) with non-thalamic HA content (ng) and levels (ng/g) in females, but not in males ($P=0.112$); these correlation coefficients were significantly different ($Z=3.124$, $P<0.002$) by the Fisher z-test. Similarly, body weight was significantly ($P<0.005$) and negatively correlated with whole brain HA content and levels in females, but not in males.

V) Correlation of MC numbers and tMH values from individual rat brains

A) Thalamic tMH and correlation with thalamic MC numbers - the tMH content of male and female thalami was lower and less variable than the HA content of these tissues (Table 10). The tMH levels showed a similar pattern (Table 10). There were no differences in either thalamic tMH levels or content between sexes by group t-test.

Table 10. TMH content (ng) and concentrations (ng/g) in male and female rat brain.

Parameter	Sex	Mean	S.E.M.	Range	C.V.(%)
Thalamic tMH (total ng)	M:	6.4	0.6	3.1-12.6	39.7
	F:	5.4	0.3	1.9-7.2	29.4
Thalamic tMH (ng/g)	M:	79.7	6.6	37.9-148.7	38.2
	F:	76.3	4.4	31.7-130.2	28.3
Non-thal. tMH (total ng)	M:	69.9	3.0	45.7-99.6	20.4
	F:	58.4*	2.9	37.4-84.0	24.3
Non-thal. tMH (ng/g)	M:	37.8	1.9	23.6-56.0	23.6
	F:	32.6*	1.6	19.8-49.9	24.8
Whole brain tMH (total ng)	M:	76.2	3.4	51.8-111.0	20.2
	F:	63.6*	3.0	43.1-91.0	23.4
Whole brain tMH (ng/g)	M:	39.6	2.0	25.5-59.0	23.3
	F:	34.2*	1.7	22.3-51.6	23.8

Frozen brains from male (thalamus and whole brain, n=21; non-thalamus, n=22) and female (n=24) rats were dissected into thalamic and non-thalamic (i.e., the remainder of the brain) portions and assayed for HA as described. Whole brain weights and whole brain HA amounts (ng) were calculated as the sum of thalamic and non-thalamic values. Whole brain HA concentrations (ng/g) are derived from whole brain weights (g) and whole brain histamine (ng). Shown are the mean, standard error of the mean (S.E.M.), range and coefficient of variation (C.V.).

* Significantly different ($P<0.05$) from male values by group t-test.

Thalamic MC numbers failed to correlate significantly with either thalamic tMH content (ng)(Fig 21) or levels (ng/g)(Fig 22) in males. In females, thalamic MC numbers also failed to correlate with thalamic tMH levels (Fig 22); however, MCs did correlate with thalamic tMH content (Fig 21) and the slope indicated that female thalamic MCs contain 0.13 pg tMH per MC, or approximately 5% of the HA content of these cells. Fisher's z transformation (Zar, 1974) indicated no difference between male and female correlations of thalamic MC numbers with thalamic tMH content ($Z=0.352$, $P>0.5$). The common correlation coefficient was significant ($z_w=0.423$, $r_w=0.399$, $P<0.01$). Actual correlation of the combined data of males and females was significant ($r=0.428$, $P<0.01$)(not shown) and indicated that brain MCs contained 0.1 pg tMH per MC (slope=0.0001, intercept=4.35 ng).

B) Non-thalamic tMH and thalamic MCs - tMH levels outside of thalamus were lower and less variable than thalamic levels in both sexes (Table 10). Neither sex exhibited any relationship between non-thalamic tMH content (Fig 23) or level (Fig 24) and thalamic MC number. There were significant differences between males and females in both non-thalamic tMH content ($P=0.009$) and levels ($P=0.043$) by group t-test.

C) Whole brain tMH and thalamic MCs - whole brain tMH levels were similar to non-thalamic levels in both sexes (Table 10). Variation of these values was similar to that of non-thalamic tMH. In neither sex did any whole brain tMH

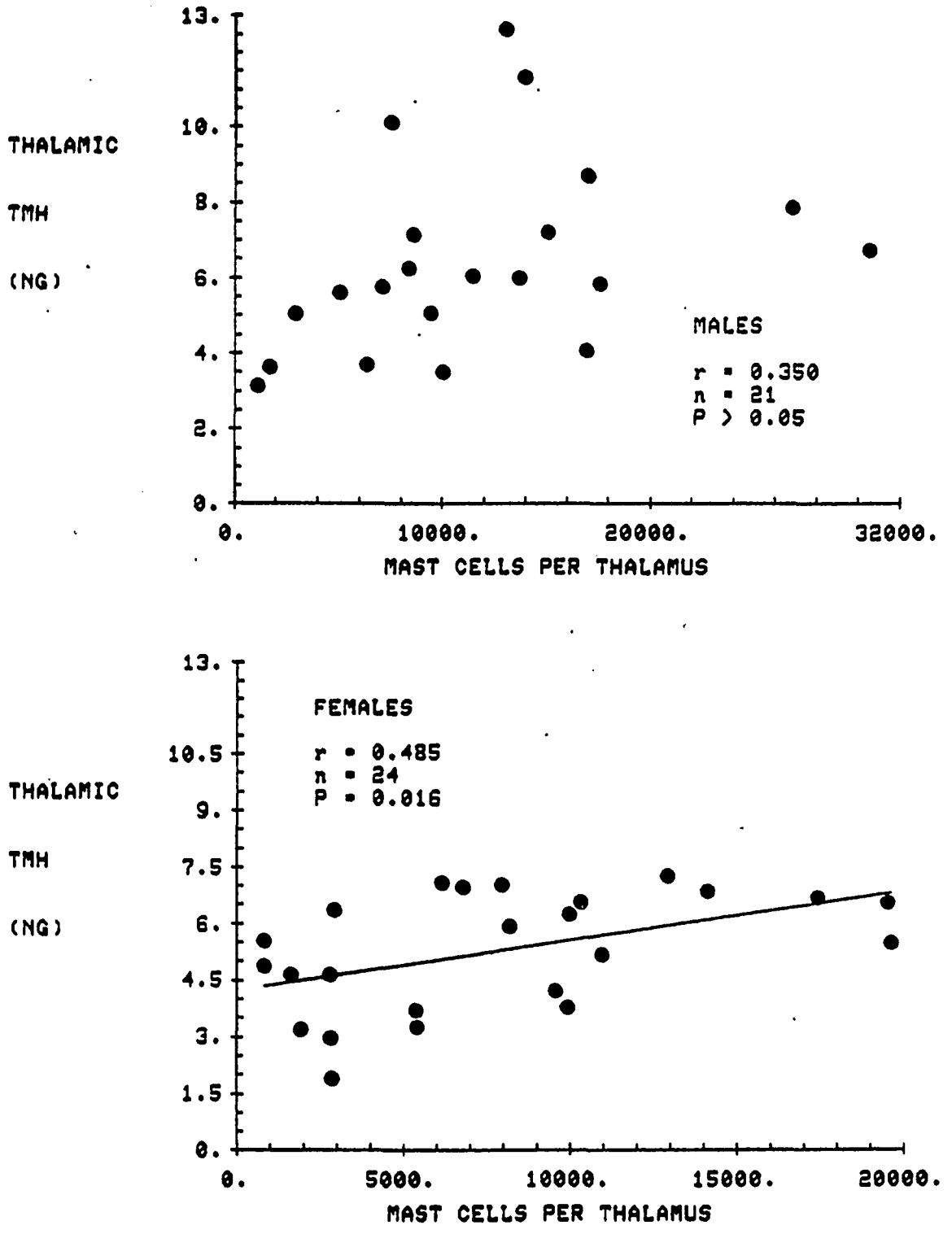

Fig 21. Correlation of estimated thalamic MC numbers with thalamic tMH content (ng) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

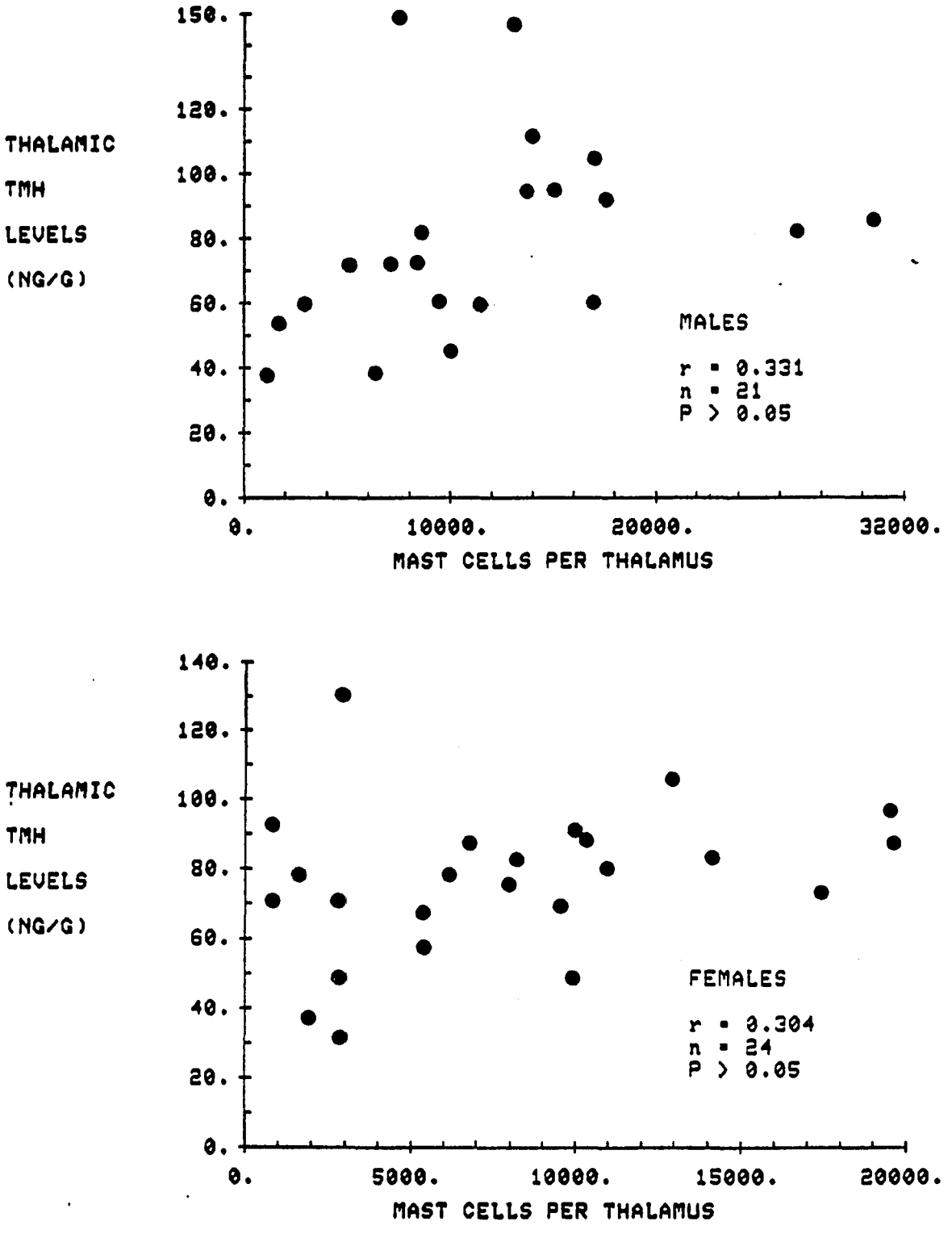

Fig 22. Correlation of estimated thalamic MC numbers with thalamic tMH levels (ng/g) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

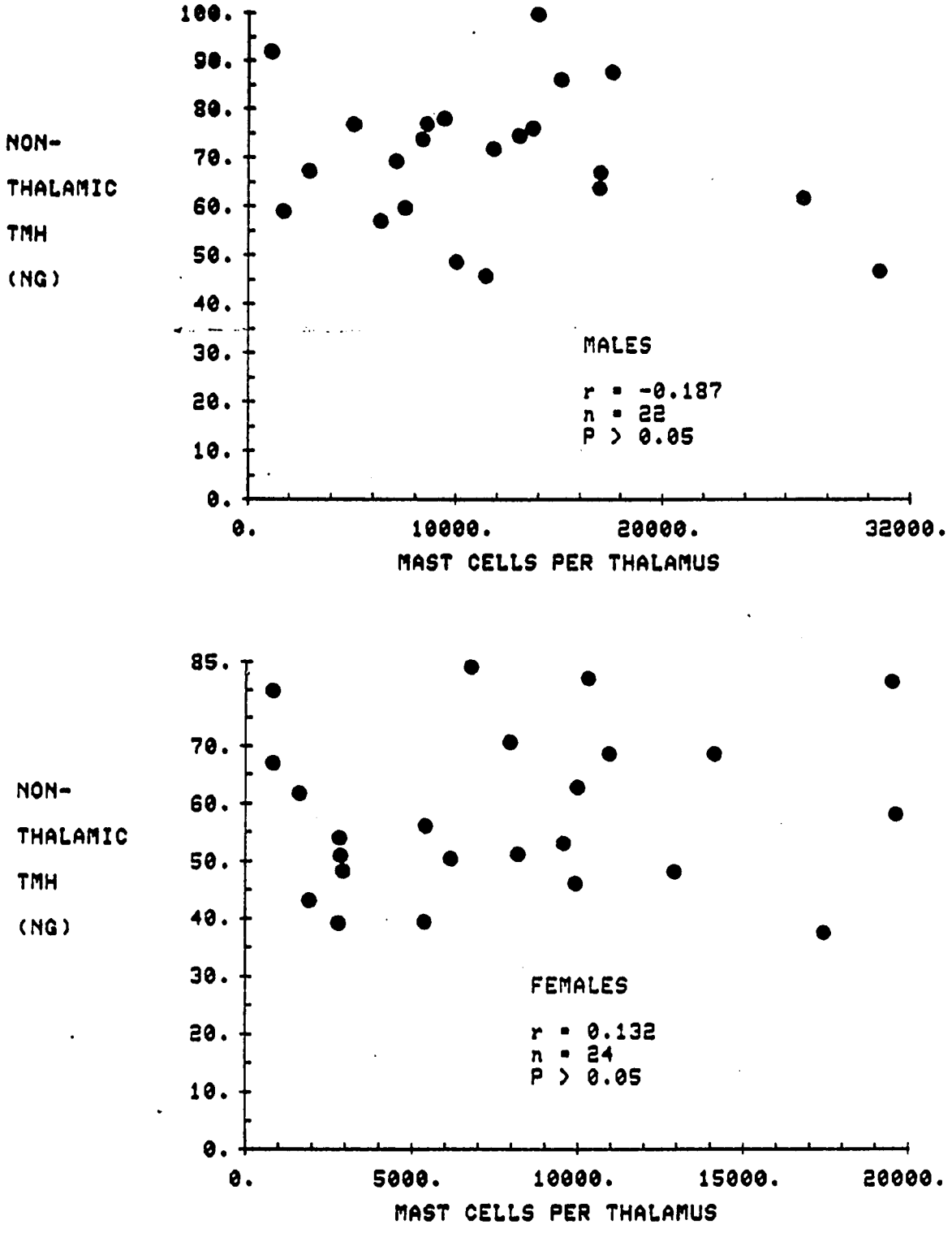

Fig 23. Correlation of estimated thalamic MC numbers with non-thalamic tMH content (ng) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

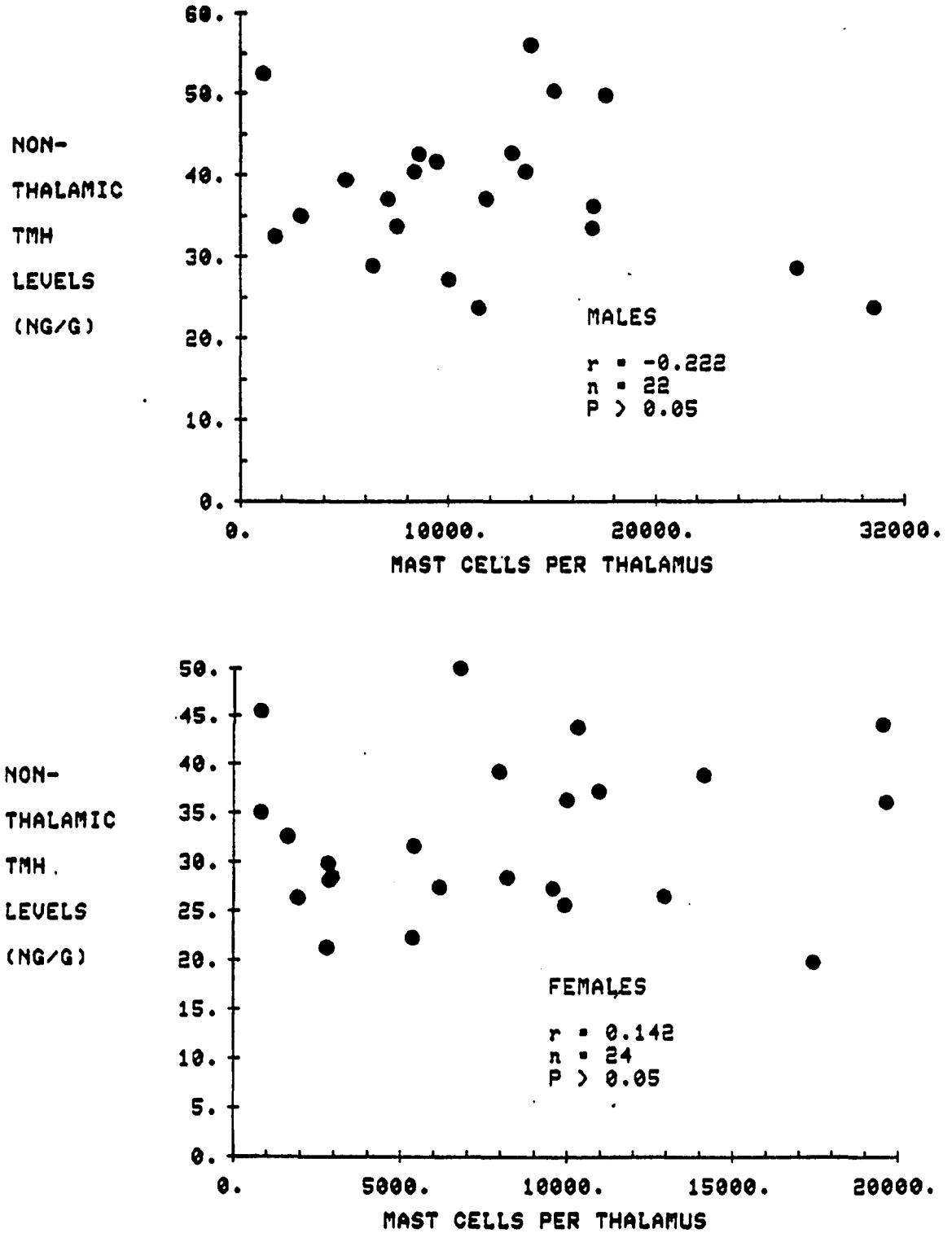

Fig 24. Correlation of estimated thalamic MC numbers with non-thalamic tMH levels (ng/g) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

parameter correlate with thalamic MC numbers (curves not shown). There were significant differences between males and females in both whole brain tMH content (P=0.008) and levels (P=0.043), but it is not known if age or weight were important (Table 10).

D) Correlations of tMH with HA from the same homogenate - thalamic tMH correlated with thalamic HA (content=Fig 25, levels=Fig 26) in males and females. Non-thalamic and whole brain tMH content and levels failed to correlate with their respective regional HA values in both sexes (males: non-thalamic content, r=-0.262, P>0.2; non-thalamic levels, r= -0.15, P>0.5; whole brain content, r=-0.11, P>0.5; whole brain levels, r=0.008, P>0.5; females: non-thalamic content, r=-0.078, P>0.5; non-thalamic levels, r=-0.099, P>0.5; whole brain content, r=0.052, P>0.5; whole brain levels, r=0.031, P>0.5).

E) Other correlations - as seen with the HA values, thalamic tMH failed to correlate with non-thalamic tMH levels or content. Thalamic tMH was significantly correlated with whole brain tMH content (r=0.486, P<0.02) and levels (r=0.427, P<0.05) in females (not shown); this relationship was not observed in males (content: r=0.329, P>0.05; levels: r=0.354, P>0.05). Comparison of the correlation coefficients by Fisher's z transformation (Zar, 1974) indicated no differences between the sexes (content: Z=0.588, P>0.5; levels: Z=0.227, P>0.5). The common correlation coefficients (of combined male and female data of thalamic tMH with whole

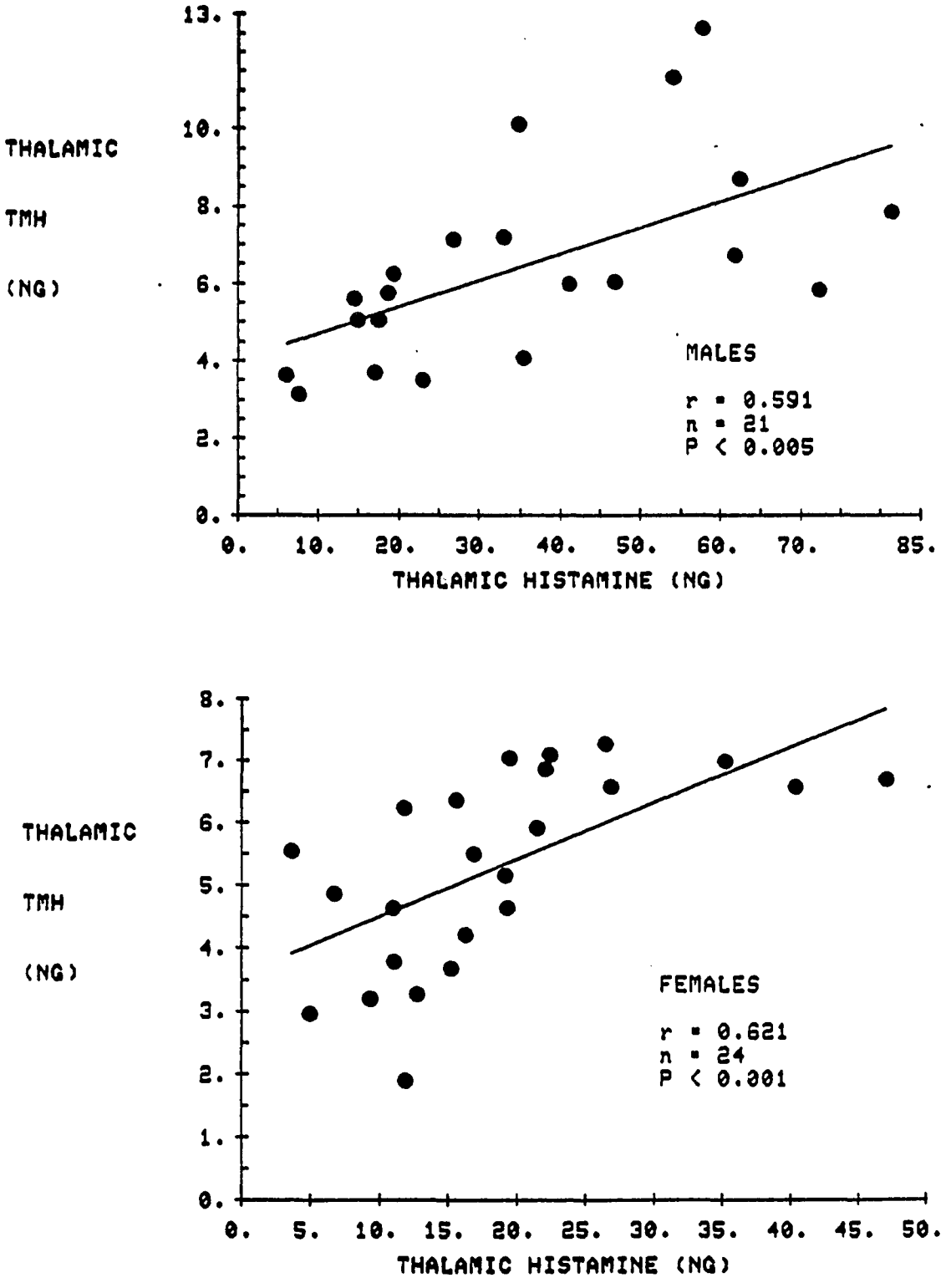

Fig 25. Correlation of thalamic HA content (ng) with thalamic tMH content (ng) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

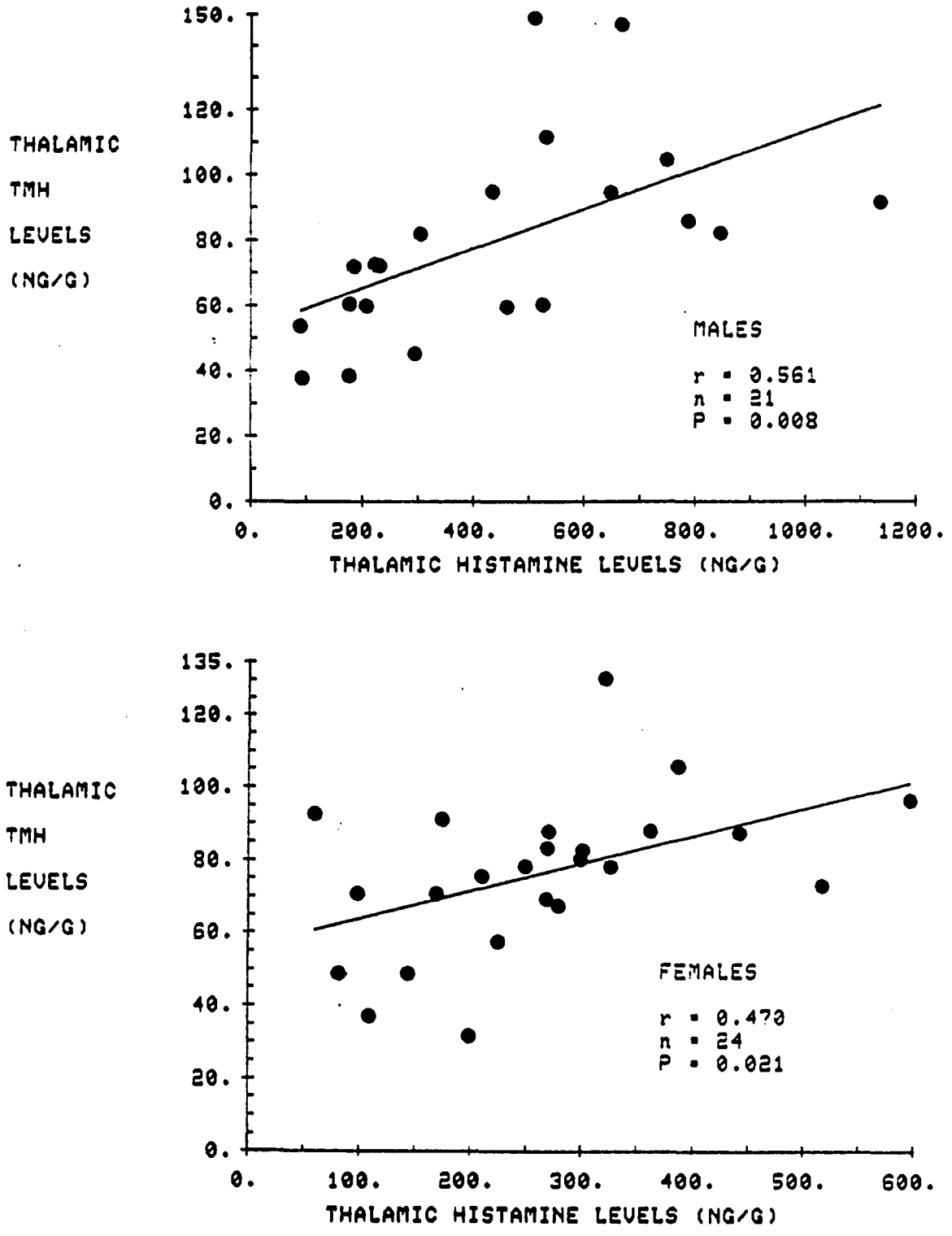

Fig 26. Correlation of thalamic HA levels (ng/g) with thalamic tMH levels (ng/g) in males (top) and females (bottom). Thalamic MC values were estimated from a single coronal cryostat section as described.

brain tMH, content and levels) were both significant (content: z_w=0.444, r_w=0.416, P<0.005; levels: z_w=0.416, r_w=0.394, P<0.01). Body weight had no relationship with any brain tMH parameter. While thalamic weight had no effect on thalamic tMH in males, females showed a significant correlation (P<0.02) between thalamic weight and thalamic tMH content (there was no relationship between thalamic weight and thalamic tMH levels in females). Non-thalamic weight was not related to non-thalamic tMH in either sex. Interestingly, while whole brain weight was not correlated with whole brain tMH in females, it was significantly and negatively correlated with both whole brain tMH content (P<0.05) and levels (P<0.005) in males.

Partial stepwise correlational analysis indicated that brain MCs were significantly correlated with thalamic HA when thalamic tMH was held constant (males: r=0.841, P<0.001; females: r=0.550, P<0.01) and that thalamic HA was significantly correlated with thalamic tMH when brain MCs were held constant (males: r=0.587, P<0.01; females: r=0.454, P<0.05). However, when thalamic HA was forced to remain constant, brain MCs showed no relationship to thalamic tMH in either sex (males: r=-0.341, P>0.1; females: r=0.111, P>0.5).

VI) <u>Differences in the regional HA content of tissue dissected while either fresh or frozen</u>

The high thalamic HA levels compared to literature values (Results section III, Table 1) and the large variation

between individuals (Table 9) prompted speculation as to whether methodological factors might account for my observations, perhaps allowing me to analyze a MC component of brain HA that might have been lost by others who used conventional methods. The most outstanding difference in my methodology as compared to previous studies of brain HA was our practice of freezing the brain solid (for histological purposes) prior to dissection. I therefore undertook a study to determine the regional HA levels in fresh (n=8) and frozen (n=9) brains of adult male rats. Dissection techniques were identical between the two groups (fresh and frozen) and all efforts were made to keep at a minimum the time between the homogenization of tissue and boiling/denaturation steps. Regions analyzed were thalamus, hypothalamus, cerebellum-brain stem and the remainder (including cortex, hippocampus, etc.). No attempt was made to determine the MC content of the brains involved in this study.

A) Treatment of regional weights - due to the extreme cold of the frozen tissue (dissected on dry ice), moisture from the air condensed and froze on the tissue during the dissection procedure. This resulted in artificially elevated regional weights of frozen tissue relative to those of tissue dissected while fresh (all regions were significantly different between the two studies by group t-test)(Table 11). In order to compare HA levels between fresh and frozen tissue, frozen tissue weights had to be corrected for the additional weight of the ice. Individual frozen tissue

Table 11. Regional tissue weights (g) in fresh and frozen brain tissues.

Region	Fresh	Frozen	Corrected Frozen
Thalamus			
mean±SEM	0.071±0.002*	0.087±0.002	0.068±0.002
CV%, n	9.6%, 8	7.4%, 9	7.4%, 9
range	0.060-0.080	0.076-0.097	0.059-0.076
Hypothalamus			
mean±SEM	0.060±0.003*	0.081±0.003	0.063±0.003
CV%, n	15.0%, 8	12.1%, 8	12.1%, 8
range	0.049-0.072	0.059-0.092	0.046-0.072
Cerebellum-BS			
mean±SEM	0.471±0.010*	0.532±0.014	0.471±0.012
CV%, n	5.7%, 8	7.9%, 9	7.9%, 9
range	0.430-0.502	0.500-0.632	0.440-0.559
Cortex-remaind			
mean±SEM	1.269±0.015*	1.387±0.025	1.269±0.023
CV%, n	3.4%, 8	5.1%, 8	5.1%, 8
range	1.217-1.325	1.264-1.484	1.157-1.358
Whole brain			
mean±SEM	1.871±0.015*	2.085±0.031	1.868±0.028
CV%, n	2.2%, 8	3.9%, 7	3.9%, 7
range	1.807-1.919	1.928-2.160	1.726-1.934

Regional brain tissue was dissected while either fresh (on ice) or frozen (on dry ice). Frozen tissue weights were corrected (Results section V-A) for the added weight due to ice condensation on tissue during the dissection procedure. The corrected frozen weights were not different from the weights of fresh tissue by group t-test.

* Significantly different ($P<0.05$) by group t-test from frozen tissue.

values in cerebellar-brain stem and cortex-remainder regions were multiplied by the means of the percentage differences between fresh and frozen tissue weights (cerebellum-brain stem=0.885, cortex-remainder=0.915). Thalamic and hypothalamic weights, being similar, were multiplied by the same value (0.778), which was the mean of their individual factors. Following these data transformations, there were no differences between the regional weights of fresh and frozen tissue (Table 11).

When comparing HA levels between fresh and frozen tissue, the values of frozen tissue were calculated using corrected weights. When comparing HA levels between the present frozen tissues and those described in section III above, non-corrected tissue weights were used.

B) Thalamic HA - the HA content of fresh thalamus was 15±2 ng while that of frozen dissected tissue was 12±2 ng, not significantly different (Table 12). The thalamic HA levels of fresh tissue were 211±34 ng/g while those of frozen tissue were 178+28 ng/g, also not different (Fig 27, Table 12). The variation in thalamic HA content (ng) and levels (ng/g) in both protocols was approximately 45% (Table 12). The present thalamic HA levels were significantly different ($P<0.0005$) by group t-test from the thalamic HA levels found in the MC-HA correlational studies described above (males: 451 ng/g, Table 9). Similarly, the present thalamic HA content (fresh: 15 ng, frozen: 11.9 ng) was significantly

Table 12. Regional HA content (ng) and levels (ng/g) in fresh and frozen dissected male rat brain.

Brain Region	Fresh/ Frozen	Mean	S.E.M.	Range	C.V.(%)
Thalamic Content	FSH:	15.0 ng	2.4	5.6-22.9	45.2
	FRZ:	11.9 ng	1.8	4.7-20.1	45.8
Thalamic Levels	FSH:	211.3 ng/g	34.3	80.7-349.6	45.9
	FRZ:	177.8 ng/g	27.8	67.8-318.0	46.9
Hypothal. Content	FSH:	9.9 ng	0.7	7.3-13.7	20.0
	FRZ:	11.1 ng	1.1	7.0-16.3	29.2
Hypothal. Levels	FSH:	169.4 ng/g	13.1	108.8-211.9	21.9
	FRZ:	177.4 ng/g	17.2	105.5-251.5	27.4
Cereb/BS Content	FSH:	9.8 ng	1.0	6.2-14.8	30.0
	FRZ:	12.0 ng	0.8	8.6-15.3	20.0
Cereb/BS Levels	FSH:	20.8 ng/g	2.3	13.2-32.6	31.1
	FRZ:	25.4 ng/g	1.4	18.3-30.5	16.5
Cort/Rmdr Content	FSH:	44.5 ng	2.1	33.8-54.9	13.6
	FRZ:	44.5 ng	2.5	34.3-54.5	15.7
Cort/Rmdr Levels	FSH:	35.2 ng/g	1.9	26.8-44.6	15.5
	FSH:	35.0 ng/g	1.8	27.9-41.1	14.2
Non-thal. Content	FSH:	64.3 ng	2.9	49.4-76.5	12.7
	FRZ:	67.8 ng	4.3	51.5-78.8	16.8
Non-thal. Levels	FSH:	35.7 ng/g	1.7	28.0-42.8	13.1
	FRZ:	37.6 ng/g	2.1	28.2-42.9	15.1
Wh. brain Content	FSH:	79.2 ng	3.4	65.9-97.0	12.3
	FRZ:	78.8 ng	4.6	60.2-96.0	15.6
Wh. brain Levels	FSH:	42.4 ng/g	1.8	35.8-52.2	12.3
	FRZ:	42.1 ng/g	2.2	33.2-50.4	13.5

Brains from adult male rats were dissected either fresh (n=8) or frozen (thalamus and cerebellum/brainstem, n=9; hypothalamus and cortex/remainder, n=8; whole brain and non-

thalamus, n=7) and assayed for histamine as described. Whole brain and non-thalamic weights and histamine amounts were calculated as the sum of individual regional values. Whole brain and non-thalamic histamine concentrations were derived from their respective histamine contents and regional weights. Shown are the mean, standard error of the mean (S.E.M.), range and coefficient of variation (C.V.). For estimated age of animals, see Table 5.

*Significantly different ($P<0.01$) from male values by group t-test.

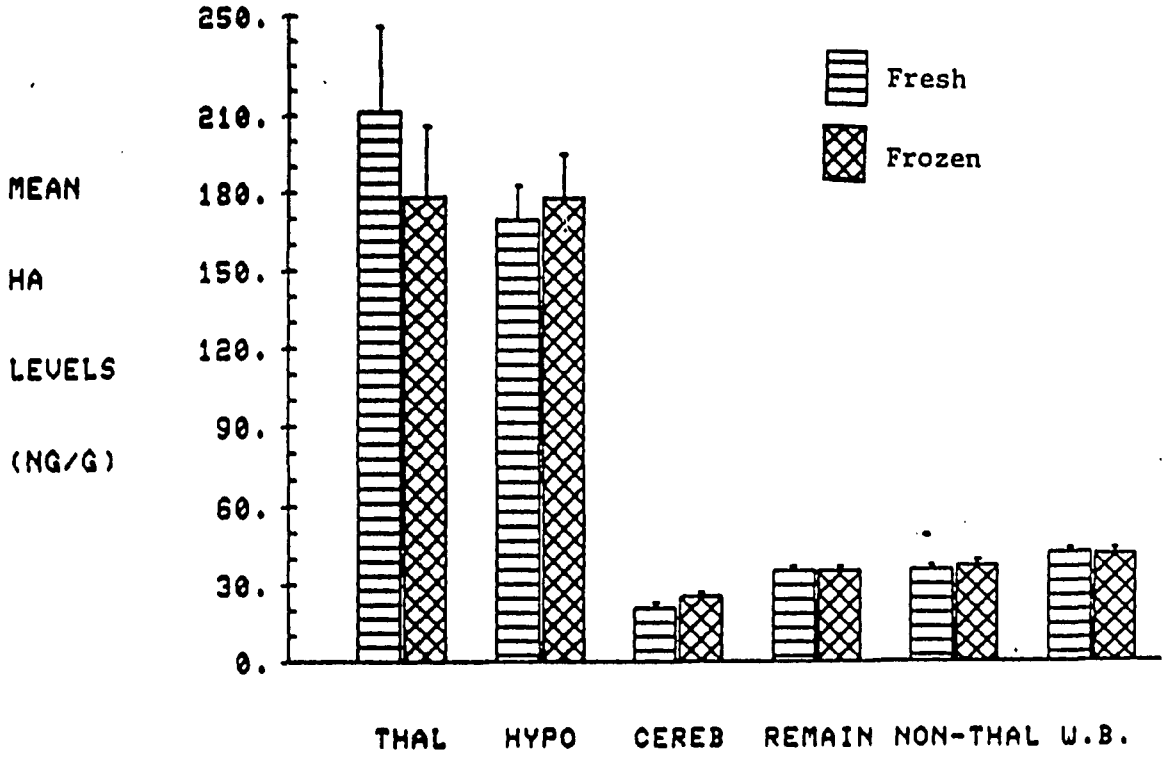

Fig 27. Comparison of the regional HA levels (ng/g) in brain tissue dissected while either fresh (on ice) or frozen (on dry ice). Shown are mean values (+S.E.M.). There are no differences between fresh and frozen HA values in any region by group t-test. There is no difference between thalamic or hypothalamic HA levels.

different ($P<0.0001$) from the values described above (males: 36.2 ng, Table 9).

C) Hypothalamic HA - the HA content (ng) and levels (ng/g) of fresh and frozen hypothalamic tissue were not significantly different from each other by group t-test (Table 12, Fig 27). There were no differences between hypothalamic and thalamic HA levels or content in either fresh or frozen tissue. The variations in hypothalamic HA content and levels were much smaller than that found in thalamus.

C) Other brain regions - in no other brain area did fresh and frozen tissue have different HA content or levels (Fig 27). Cerebellum-brain stem had the lowest HA content and levels of all regions examined while cortex-remainder had HA levels similar to non-thalamic and whole brain regions (obtained by addition of regional brain values, Table 12). With the exception of fresh cerebellar-brain stem tissue (CV=approximately 30%, Table 12), the coefficients of variation of all areas were much lower (approximately 15%, Table 12) than that seen in thalamus (approximately 45%, Table 9). Interestingly, although there were no differences between the present frozen non-thalamic HA values (content: 68 ± 4 ng; levels: 38 ± 2 ng/g, Table 12) and those observed during the MC-HA study (males, content: 75 ± 4 ng; levels: 41 ± 2 ng/g, Table 9), the present whole brain HA values (content: 79 ± 5 ng; levels: 42 ± 2 ng/g, Table 12) were significantly lower by group t-test from the previous whole brain values

(males, content: 112±7 ng; levels: 58±3 ng/g, Table 9) in both content ($P<0.0005$) and levels ($P<0.001$).

I) Characterization of rat brain MCs

A) Toluidine blue and Astrablau - the finding that brain MCs are capable of staining metachromatically with toluidine blue following aldehyde fixation below pH 4 (Fig 11; Fig 13, upper left) indicates that these cells contain an acid glycosaminoglycan that is substantially similar to that of peripheral MCs. MMCs do not stain at low pH (Enerback, 1966) and require enzymatic treatment in order to bind toluidine blue following aldehyde fixation (Wingren and Enerback, 1983). However, brain MCs stained variably at lower pH. This might be the result of either too short a staining time or a slightly different granular proteoglycan that is not as highly sulfated as that found in MCs outside the brain. The use of alchohol to confer specificity to the stain ("true metachromasia"; Schubert and Hamerman, 1956), i.e., to highlight the deep metachromatic staining of the MC from the lighter orthochromatic staining of background elements such as neuronal cell body nuclei, is further evidence of the similarity of brain MCs to their peripheral counterparts.

That brain MCs also stain with Astrablau under conditions precluding staining of other cellular elements (Fig 13, lower right) is further evidence of the presence of a highly sulfated glycosaminoglycan in these cells. While other reports of the staining of brain MCs with Astrablau have appeared (Kruger, 1974; Edvinsson et al., 1977), these authors did not present pictures, failed to show that these cells were the same as those that stain with toluidine blue

(Fig 13, lower left) and did not give adequate details of methods used.

According to these results, it is now possible to classify rat brain MCs as being in the same histochemically identifiable family as the "typical" MC.

B) OPT fluorescence and HA - the finding that rat brain MCs fluoresce following gaseous OPT treatment is unequivocal evidence that brain MCs contain HA (Fig 13, upper right). Edvinsson et al. (1977) identified HA in brain MCs of several species (including rat and primate) using OPT dissolved in the paraffin used for embedding. They also performed spectrofluorometry on the samples and found that the wavelengths corresponded to that of authentic HA. However, the only photographs shown were of meningeal MCs; none were shown from neuropil. While OPT is also capable of complexing with other amines and amino acids, none of these substances (with the exception of HA) have been identified as being present in peripheral MCs.

The OPT-reactive cells are mostly the same as those that stain with toluidine blue (Table 4, Fig 14). 80% of the observed cells stained positively with both methods. The 15% that stained only with toluidine blue most likely showed this behavior due to the physical characteristics of the reagents used. OPT and water vapor may not have fully penetrated the section in the short time allowed for the reaction to take place. Toluidine blue, in solution, acted for a much longer time and penetrated the entire section, staining all the

cells. In several cases, adjacent mast cells in the same section reacted differently, one visualized only with toluidine blue and the other with both procedures. In all such cases, careful focusing indicated that the two cells were at different depths within the section, with the OPT-negative cells some distance from the exposed surface. We have no explanation for the 5 percent of cells that were OPT-positive and toluidine blue-negative, although they might be vascular elements (see Fig 14).

The immunohistochemical visualization of HA in neuronal elements within the CNS (Introduction section III-B-ii) is a crucial piece of evidence needed to show that this amine functions as a neurotransmitter in brain. That these reports did not mention brain MCs (Wilcox and Seybold, 1982) or failed to visualize them in any area in brain except median eminence (Panula et al., 1984) and the ventral surface of hypothalamus (Steinbusch and Mulder, 1985) is puzzling. The serum used by Wilcox and Seybold (1982) was not able to visualize HA in peripheral MCs (personal communication, Dr. L.B. Hough). Panula et al. (1984) were able to intensely stain peripheral MCs, leaving out the possibility that their observations of MCs in the median eminence MCs might be artifactual and that their antibodies were not able to bind to MC HA at all. While it is possible that there is a difference in the manner in which HA is bound to MC granules and neuronal elements, the ability of Panula et al. to visualize MCs in peripheral organs precludes the possibility

of a specificity in the antibody recognition of the HA binding site in MCs relative to neurons.

C) Formaldehyde fluorescence and 5HT - the autofluorescence of brain MCs in experimental control sections (Results section I-D) precludes identification of 5HT in these cells. Further complicating this experiment was the rippling of the tissues upon counterstaining with aqueous toluidine blue. However, multiple photographs of toluidine blue-stained sections at different levels of focusing establishes that there is a one to one correspondence between autofluorescing MCs and those that stained with toluidine blue. In addition, as there was no observed fluorescence from neurons, it is likely that the procedure used was not working properly, and any 5HT in thalamus (either in MCs or neurons) was not visualized. Thus, the question of whether brain MCs contain 5HT remains unanswered. Edvinsson et al. (1977) reported the visualization of 5HT in brain MCs of several species, including rat. However, they only showed meningeal spread tissue. There are no other reports of the successful histochemical visualization of 5HT in these cells.

D) Heterogeneity of brain MCs - just as MCs in peripheral organs have been described to be different between tissues and species (Introduction section I-F-ii-6), there have been reports of histological and morphological differences between brain MCs. Flood and Kruger (1970) observed a continuum of hedgehog brain MCs by electron microscopy, with those near the vasculature having a distinctly different granular

structure than those further away. Cammermeyer (1972,1973) described a similar phenomenon in monkey brain using light microscopy, again concerning MCs lying at different distances from vasculature (Cammermeyer, 1973). Both authors attributed their observations to different activities of MCs, with those further away from blood vessels containing fewer and less dense granules. This supposes that brain MCs are physiologically active in-vivo. It is also possible that there are different types of MCs resident within similar areas of brain, as has been described by Ibrahim (1974). Based of histochemical and electron microscopic data, Ibrahim (1974) proposed that rat brain contains two types of MCs. According to his hypothesis, type I cells corresponded to classical MCs, being round to oval, metachromatic after toluidine blue staining, situated near blood vessels and located primarily in thalamic areas (a distribution similar to that observed by others, see Introduction and Results). Another cell, called type II, was more elongated and spindle shaped, not metachromatic, was associated with blood vessels, contained lipid rich granules of a more variable size than type I cells and was distributed throughout brain. Such a classification has not been widely accepted, as Kiernan (1976) described equivalent observations and termed them pericytes. In a subsequent report, Ibrahim et al. indicated that type II cells were metachromatic to normochromatic and termed them neurolipomastocytoid (NLM) cells (1979a). Should such cells exist, a difference in the acidic mucopoly-

saccharide content of the granules might account for the difference in the metachromatic behavior and raises questions as to whether these cells might contain HA or other mediators. However, this investigator has not observed any cells in rat brain that might correspond to Ibrahim's type II classification. As formol-alchohol fixed tissue was reported to prevent visualization of these cells (Ibrahim, 1974), it is possible that methodological differences are the basis of the discrepant results. These cells were reported to degranulate in response to compound 48/80 (Ibrahim et al., 1979b).

II) Anatomical localization of rat brain MCs

A) A-P distribution of brain MCs

i) Regional distribution - as discussed in the introduction (section II-B-vi), brain MCs in the rat have been observed to have a primarily thalamic distribution, with a few being observed in cortical areas. The present results have confirmed these findings, as greater than 98% of brain MCs were observed in thalamus, with the remainder residing in cortex (Results section II-A). With the exception of one report (Edvinsson et al., 1977), there have been no histochemical reports of MCs being found in hypothalamic areas. However, the presence of MCs in this area has been inferred by biochemical data and these cells have been observed immunohistochemically in the median eminence (Introduction section III). One possible reason for these

discrepancies is the method of brain removal. Unless one takes great care during the removal of the brain from the cranium, the pituitary and stalk remains in the base of the skull. Under these conditions, the median eminence might remain behind. Should this area be the only non-thalamic region to contain appreciable numbers of MCs, then the methodological differences in dissection techniques would account for differing reports of hypothalamic MCs.

ii) A-P distribution of MCs within thalamus - MCs exhibit a striking A-P distribution within thalamus (Fig 15,16). This pattern was present in all animals regardless of their total thalamic MC numbers. A somewhat similar wave of MCs within thalamus was shown by Persinger (1977b) in 21 day old rats.

B) Thalamic nuclear distribution of rat MCs - within thalamus, rat brain MCs display a distinct nuclear distribution (Table 6). Males and females have similar nuclear distributions, with large numbers of cells being found in the ventral nuclear complex (i.e., the ventral and ventral dorsal nuclei), medial dorsal, lateral, and paraventricular nuclei. Estimates of the volumes of 14 thalamic nuclei (those of Table 6 plus the medial geniculate, lateral posterior, ventral anterior, and reticular nuclei; obtained by cutting out and weighing areas delineated on copies of individual atlas pages; Pellegrino et al., 1979), showed that 89 percent of female thalamic mast cells and 95 percent of male thalamic mast cells were confined to only 60

percent of total thalamic volume. Thus, mast cells were unevenly distributed within thalamus (females: $X^2=35$, $P<0.0001$; males: $X^2=51$, $P<0.0001$). However, within these nuclei, a significant correlation (females: $r=0.898$, $P<0.0001$; males: $r=0.932$, $P<0.0001$) was found between mast cell numbers and the volume of these nuclei, indicating that the total density of the individual nuclei (total MCs per nucleus/total nuclear volume) within this group was constant. A similar nuclear distribution of thalamic MCs in three adult albino male rats was previously reported (Dropp, 1972), and concentrations of MCs within specific thalamic nuclei have been reported in 21 day old rats (ventral, anterior ventral, reticular and parafascicular nuclei; Persinger, 1979), and hedgehog (medial dorsal nucleus; Campbell and Kiernan, 1966: habenular, medial dorsal, lateral and geniculate nuclei; Kruger, 1970). However, these authors did not present quantitative data and therefore were not able to ascertain the contribution of thalamic nuclei to total thalamic MC numbers.

C) Localization of MCs in brain: perivascular or neuropil - the question of whether brain MCs are completely associated with vasculature of brain has been controversial. Should they be free of vasculature and resident in neuropil tissue, they might be capable of playing a role in neuronal physiology. While numerous authors have described MCs as being perivascular (Kelsall and Lewis, 1964; Kelsall, 1966; Dropp, 1972; Kruger, 1974; Ibrahim, 1974; Barron et al., 1974),

several reports have indicated that despite attempts to find vascular connections, at least some brain MCs lie completely within the brain parenchyma or neuropil (Flood and Kruger, 1970; Kiernan, 1971,1976; Kruger, 1970; Dropp, 1976; Edvinsson, 1977; Cammermeyer, 1973). Flood and Kruger (1970) found that although perivascular MCs in the hedgehog were surrounded by a basement membrane that separated them from both endothelia and neuropil, other cells, resident within neuronal parenchyma, were completely without this membrane and so were hypothetically capable of interaction with surrounding neurons.

Although some MCs in rat thalamus showed no association with blood vessels, the methods used precluded the absolute determination of whether these cells are fully resident within brain parenchyma. Vascular connections might have been outside the plane of the section. To be certain of the vascular status of brain MCs would have required serial sectioning of the entire thalamus and examination by light and EM microscopy, a technique which was not performed.

D) Possible reasons for the localization of MCs within thalamus

i) Blood flow and vasculature of thalamus - as the MCs of brain have a significant perivascular localization and the amines released from peripheral MCs upon stimulation have potent actions on vascular endothelium (Gross, 1982), it seems possible that a vascular factor (i.e., degree of vascularization, permeability of the blood-brain barrier or

degree of blood flow) distinct to the thalamus might be implicated in the thalamic distribution of these cells. However, regional blood-flow and blood transport kinetic studies using different tracers have indicated that while thalamic blood-flow and glucose utilization are on the high end of the numerous brain regions examined, these values are not unique, and there are numerous areas that have similar values but contain no MCs (Reivich et al., 1969; Marcus et al., 1976; Sokoloff et al., 1977; Sakurada et al., 1978; Sokoloff, 1981a and b; Pardridge et al., 1982). There is no evidence that thalamus has either more vasculature or a greater degree of permeability than other brain regions. Thus, if vascular parameters are functional in affecting the distribution of brain MCs, these parameters are of a more subtle nature than those examined to date.

ii) Functions of thalamus - a majority of thalamic nuclei receive input of a sensory nature from numerous afferent systems and in turn project to both specific and diffuse areas of cortex and the striatum. In turn, any thalamic region that projects to cortical and striatum regions receives input from those regions that are of the same nature as the original output (specific or diffuse)(Zeman and Innes, 1963; Jones, 1981,1983). Based on these observations, most thalamic nuclei have been considered to function as somatosensory relays, conveying and integrating sensory information to cortical areas and in turn receiving input

from these higher areas, allowing for the modification of the ascending input.

Most of the thalamic nuclei that contain large populations of MCs have been implicated in specific sensory pathways. The ventral thalamic complex, which contains the ventral, ventral medial, ventral lateral, ventral posterior and ventral anterior nuclei, has been shown to receive input from the spinothalamic tract (gracilothalamic and cuneo-thalamic tracts), from the medial and trigeminal lemnisci and from cerebellar nuclei. Additional input has been observed from the solitary nuclear complex (implicating taste functions) and inferior colliculus and cochlear nuclei (implicating auditory functions)(Zeman and Innes, 1963; Jones, 1981,1983). Ventral nuclei have been implicated in the conveyance and modification of noxious stimulii (Guilbaud, 1980; Dickenson, 1983; Casey and Morrow, 1983) and in the control of non-stereotypical posture and locomotion behavior (Starr and Summerhayes, 1983). Other thalamic nuclei that contain significant MC numbers have been shown to be involved in hearing (medial geniculate: Jones, 1981,1983; Weiner and Morest, 1983), vision (lateral geniculate nucleus, with inputs from superior colliculus and pretectal areas: Jones, 1981,1983) and taste (medial dorsal nucleus, with inputs from the lateral olfactory tract and the amygdala: Zeman and Innes, 1963; Jones, 1981,1983).

Despite the sensory functions of the thalamic nuclei in which brain MCs are found, evidence relating thalamic MCs to

thalamic sensory processing is not clear. Changes in the light/dark cycles of young rats claimed to result in significant changes in MC numbers within the lateral geniculate (Mares et al., 1979). However, these authors indicated that their "MCs" could only be visualized with toluidine blue at pH's greater than 6.7; below this pH, these cells could not be observed. Thus, their findings, though intriguing, cannot be considered valid. Handling of neonatal rats resulted in a significant (50%) decrease in MC numbers in the adults (Persinger, 1980). Whether these changes in MC numbers were directly due to changes in neuronal function or were secondary to vascular changes is not known.

iii) Possible of role of peptide factors in the thalamic localization of rat MCs - evidence indicates that as neonates, rat brain MCs are primarily localized to meningeal membranes surrounding frontal cortex, and as the animal matures, this distribution drastically alters as meningeal MC numbers rapidly decline and there is a change in their localization to thalamic areas (Introduction II-D). This process is essentially completed within in a five day period, ending by the time the rat is three weeks old. As there are no data concerning the ontogeny of the vascularization of the rat thalamus, the cause(s) of this change can only be speculative. Whether the dural MCs shift their localization, i.e., show motile behavior, or whether new MCs grow in thalamic areas from stem cells in-situ in not known. However, MCs were observed to increase in number in

peripheral nerve trunks of neonatal rats injected with nerve growth factor (NGF), a peptide factor known to promote neuronal growth (Aloe and Levi-Montalcini, 1977). This effect was specific for MCs and was not secondary to increased neuronal development. Should parts of thalamus be uniquely capable of producing this peptide or similar one, MCs in young rats might grow within this area from undifferentiated stem cells. Whether such precursor cells are present in other brain regions or whether any such peptide might be inactive or unable to reach other regions cannot be speculated upon.

MCs may not only be involved in the control of vasculature (at least in pathological conditions), but possibly in its growth. Tumors are generally characterized by an increased degree of vascularization, and increased numbers of MCs have been observed to precede such growth (Kessler et al., 1976). Tumor derived peptides (structures unknown) and two artificial peptides were shown to have potent chemotactic activities on rat peritoneal MCs, which have not previously been shown to have significant motile capabilities (Poole and Zetter, 1983). Should thalamic areas be capable of the release of similar peptides during the period of the distribution change of brain MCs from dural membranes to thalamus, then this might account for not only the change in distribution but for the localization of MCs to thalamus at later periods.

III) Quantification of rat brain MCs

A) Total thalamic MC numbers - rat brain MCs varied over an order of magnitude in all experiments performed (Table 5). This degree of variation has been observed previously (see Introduction section II-B-v). However, with the exception of one study (Dropp, 1972), quantification of MC numbers has been incomplete. Further, the numbers of brain MCs observed presently are considerably higher than those observed in adult rats by other investigators (see Introduction section II-C-ii).

At present, there is no simple explanation for this extreme variation. As discussed, MCs have been implicated with vascular elements. However, studies have indicated no extreme variations in either the extent of vasculature or blood flow within the thalamus. While circannual variations in MC numbers might exist (Kruger, 1970), all animals in these studies were sacrificed within a relatively short time of each other. Age has been reported to affect MC numbers, decreasing them with age (Dropp, 1976; Ferrer, 1979; Persinger, 1981), yet all animals within an experimental group were of similar ages. As discussed, sensory environmental factors might change MC numbers, but all animals in these studies were kept in similar surroundings. Rat dural MCs have been reported to undergo circadian rhythm (Karedina and Dovbysh, 1981) and a similar rhythm was observed in skin MCs of mice (Polat, 1980), yet all animals were sacrificed within a 4 hour morning period, and there was

no relationship between time of death and MC numbers. What is left is that there must be some intrinsic factor that governs the number of MCs within an individual. Persinger (1979,1980) found that greater than 50% of the variability in MC numbers between individuals was of intra-litter origin, i.e., that variations between littermates accounted for at least half of the observed variation.

B) Left-right asymmetries in thalamic MC numbers - one of the two quantification studies performed (Results section III-B-ii) indicated that there was a significant asymmetry in MC numbers between left and right sides of brain in both males and females. As this difference could not be localized to any specific coordinate(s), the asymmetry was spread over the entire length of the thalamus. This result was not replicated in a second group of animals (Results III-C). The failure to repeat this finding is most probably a function of the extreme variability in MC numbers inherent in these animals and does not indicate that the observed asymmetries in the first group was artifactual. ANVAR of the combined data from both sets of animals indicated that a significant ($P<0.005$) side difference in MC numbers remained.

While lateralization of neurotransmitters and cerebral function has been documented (Glick and Ross, 1981; Glick et al., 1982), the asymmetry in MC numbers is the first such observation of a non-neuronal cell in the CNS. While observations of left-right differences in MC numbers in other species has been mentioned (Kruger, 1970; Cammermeyer, 1973),

these studies were not quantitative in nature. The present finding is the first documentation of such an asymmetry in the rat.

C) Sex differences in thalamic MC numbers - a sexual dimorphism in MC numbers was observed in males and females of the first study performed (Results section II-B-iii). Females had more cells than males and this difference was spread over the entire thalamus. While a trend towards females having more MCs than males was apparent in study 2 (Fig 16) and the mean MC number for females was higher (Table 5), this difference was not significant by ANVAR (Results section III-C). When comparing the two data sets, it was determined that a decrease in MCs in the left side of females of the second set (age matched) relative to the first set was responsible for the lack of a significant sex difference in the brain MC numbers of animals used in study 2. ANVAR of the combined data from studies 1 and 2 indicated that a significant ($P<0.02$) sex difference in brain MC numbers remained.

The age of the animals in study 2 was approximately 12.2 weeks for both sexes. While the ages of the animals from study 1 are not known, ages can be estimated from weight/age curves supplied by suppliers of the animals (Fig 28). According to these charts, males of study 1 averaged 9.5 weeks while females averaged greater than 16 weeks of age. While there were no differences in total MC numbers (by sex) between the two experiments, the MC numbers in the left side

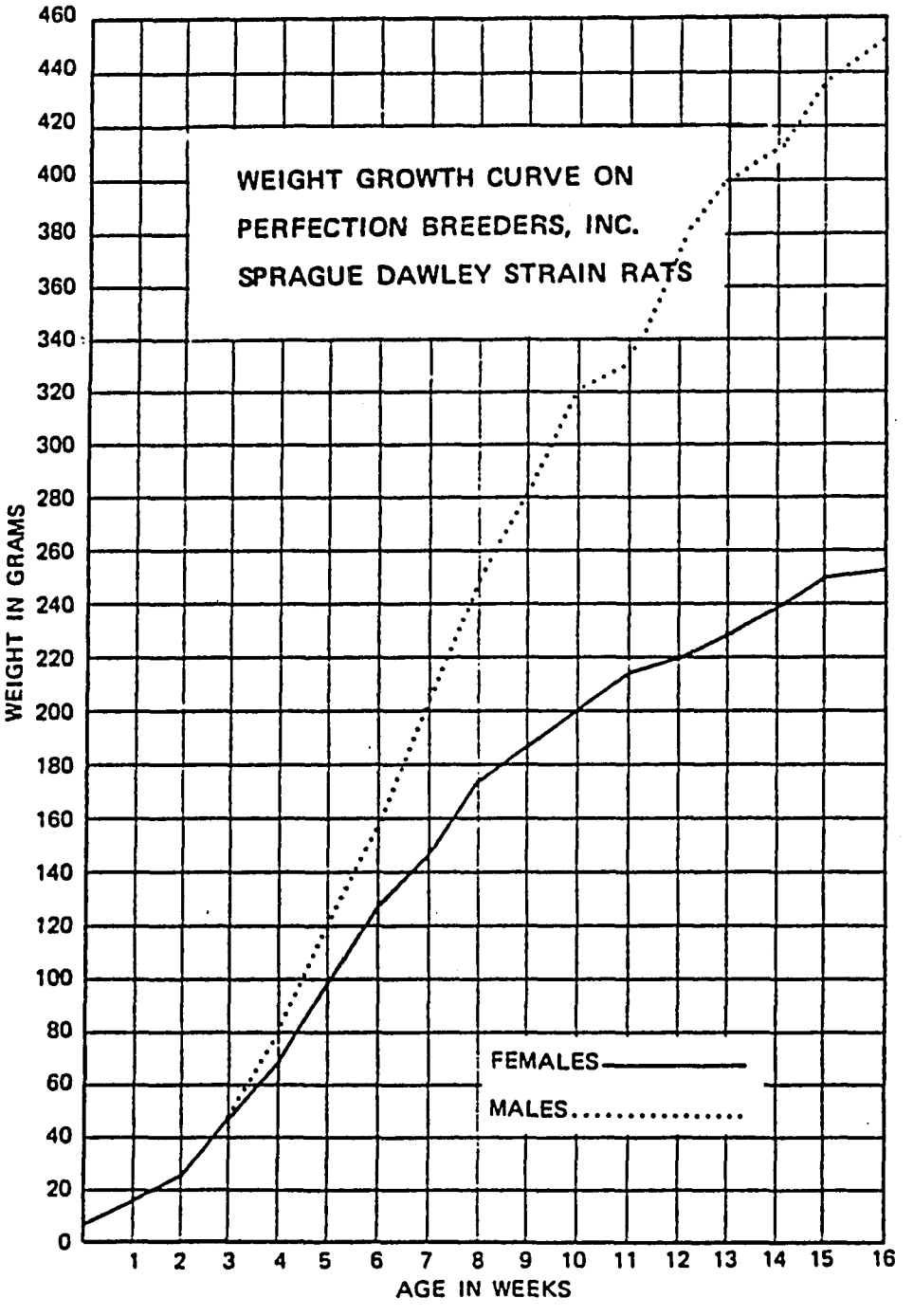

Fig 28. Comparison of age and weights in male and female Sprague-Dawley rats. The information in this chart is for comparison purposes only and is not to be considered as anything more than a graphical presentation of general population trends.

of females were higher (by ANVAR) in older animals (study 1) than in younger ones (study 2)(Table 5). There were no differences between study 1 and study 2 in the MC numbers in the right sides of females or in males (either side). Previous studies have shown that rat brain MC numbers decrease with age (Dropp, 1976; Ferrer et al., 1979; Persinger, 1981). This is in contrast to the present findings which suggest an increase with age (it should be noted that there is a general increase in MC numbers in peripheral tissues with age - rat: Padawer and Gordon, 1956; Jaques and Reugg, 1970; Wilhelm et al., 1978; Berlin and Enerback, 1978 - monkey: Schwartz et al., 1975). This discrepancy is not presently understood.

The trend in study 2 for females to have more MCs than males and the significant sex difference that remained after the two studies were combined makes a true sex difference in rat brain MC numbers highly likely. A sex difference in thalamic MC numbers was observed in hedgehog (females>males; Kruger, 1970) in a few animals of unknown ages. No difference in numbers between males and females were observed in the area postrema of cats and monkeys (Cammermeyer, 1972) or in thalamus of rats (Dropp, 1976). My observation of a sex difference in rat brain MC numbers is the first such finding in this species.

IV) Relationships between thalamic MCs, brain HA and tMH

A) Estimation of thalamic MCs from single sections - the distinct characteristics of rat brain MCs (i.e., their thalamic localization, the precise A-P distribution within thalamus and the extreme variability between individuals) presented an opportunity for the estimation of thalamic MC numbers while at the same time preserving the tissue for biochemical analysis. This allowed for the first direct investigation of the relationship between brain MCs and brain amine levels. The variability allowed for a wide range of values to be involved in the correlations of both MCs per coordinate versus total MCs per thalamus and of thalamic MCs versus thalamic HA/tMH. This wide range in MC values increased the possibility in obtaining a significant correlation.

There are several sources of error inherent in the estimation of thalamic MC numbers by this method that, while not negating the validity of the measurements, must be kept in mind during the analysis. First, the error in the direct MC quantification necessary for the correlations of MCs per coordinate versus MCs per thalamus (Tables 3,7,8), though small (approximately 10%), contributed to the overall error. Second, correlations and fitted line parameters are population estimates and cannot take into account individual variability, and thus the estimations of thalamic MC numbers cannot to bc taken as absolute values. However, the wide range of MC numbers obtained by direct quantification decreased the error inherent in estimation of MC numbers.

Further, errors in the evaluation of thalamic MC numbers should be random, and increasing the number of animals used in the analysis (n) decreases the possibility that the estimated values obtained from the sample were not reflections of the overall populations. That the probability values obtained in all correlations of MCs per coordinate versus total MCs and MCs per thalamus versus thalamic HA (Results section III-A,B) were so significant is further indication of the validity of these analyses.

B) Regional HA values and correlations with thalamic MC numbers

i) Thalamic HA levels - the HA levels (ng/g) in both males and females (Table 9) were higher than those seen in previous reports of the regional distribution of brain HA (Table 1). Further, the variation of thalamic HA levels in these animals was of a similar magnitude as that of the estimated thalamic MC numbers (Table 5) and was higher than that of non-thalamic tissue. With the exception of the studies of Oishi et al. (1983,1984), the variation in thalamic HA levels found by previous studies has not been different from that of other brain regions. Oishi found a pattern of variability similar to the present data, with thalamic HA being more variable than other brain regions (1983, CV%=66.5%; 1984, CV%=54%).

ii) Non-thalamic and whole brain HA values - non-thalamic HA values were both lower and less variable than those of thalamic HA (Table 9). Whole brain HA levels were in the

high end of rat brain HA levels previously reported (40-60 ng/g: Green, 1970; Hough and Green, 1984). Although there were no significant sex differences in the levels of non-thalamic and whole brain tissues, it is interesting that while females had higher mean non-thalamic levels than males, it was the males that had higher whole brain levels. This was due to the higher thalamic levels of HA in the males. It seems possible that the unusually high thalamic HA levels assayed in this series of animals is responsible for the slightly elevated whole brain HA levels in these animals (approximately 55 ng/g, Table 9) relatively to values found previously by others (40 to 45 ng/g).

iii) Correlations of thalamic MCs and regional HA values

1) MCs versus thalamic HA - correlations of thalamic MCs with thalamic HA were highly significant for both sexes (Results section III-B) and indicate that thalamic MCs contained 2.5 and 1.3 pg HA per MC for males and females, respectively. It is clear that the HA concentrations within these cells is much lower than that found within peritoneal MCs. Low values for the HA content of peripheral MCs have been found in other species and the heterogeneity of MCs between tissues of the same species lends support to this finding. The lower HA content of these cells might have been predicted as, assuming that these cells contained similar HA levels as peripheral MCs, only 300 cells would be needed to account for the entire thalamic HA content (see Introduction section III-D) and our measurements of thalamic MC numbers

indicate far greater numbers are present (200 to 32000; Table 5). Perhaps this lower HA content accounts for the difficulty that previous investigators have had in visualizing this amine in brain MCs (Hakansson, 1970; Ibrahim, 1974; El-Akad and Brody, 1975).

Extrapolation of correlation curves to the Y-axis allowed for the estimation of thalamic non-MC HA content and levels. Non-MC HA content (ng) evaluated in this manner was 6.8 and 8.5 ng for males and females respectively. This low HA content is in line with the relatively sparse histaminergic innervation of thalamus evaluated by immunohistochemistry (Introduction section III-B-iii). Interestingly, the non-MC HA levels measured in this manner were 91 and 143 ng/g for males and females respectively, similar to values shown in previous reports (Table 1).

2) Thalamic MCs versus non-thalamic and whole brain HA - as discussed (Results section III-C), thalamic MC numbers failed to correlate with both non-thalamic HA content and levels in males. This is in line with the hypothesis that brain MCs contain HA and, while contributing to HA levels within thalamus, do not do so in areas outside the region where they are situated. Females were not as clear cut in their results, with the correlation of thalamic MCs versus non-thalamic HA content just missing significance (Fig 19) and the correlation of MCs versus non-thalamic HA levels just attaining the significance level at P=0.049 (not shown). However, comparison of the male and female correlations of

thalamic MC numbers with non-thalamic HA levels by z transformation analysis showed no difference between them, and the common correlation coefficient was not significant (Results IV-C). Thus, thalamic MC numbers are not related to non-thalamic HA levels.

Another possibility for the less distinct results from females is that estrous might have played a role in the HA content of brain MCs. Sexual cycles have been shown to have highly significant effects on both numbers and HA content of female rat peritoneal MCs (Jaques and Ruegg, 1970). While it is unlikely that estrous played any significant role in the variability of brain MCs (although females invariably had a larger coefficient of variation in their MC numbers than did males, Table 5), the possibility does exist that HA levels of these cells might have been altered. Such an occurrence might then alter MC-HA correlations both in thalamic and non-thalamic areas. Of interest are the observations that while no differences in non-thalamic HA were observed between males and females, females had a significantly lower thalamic HA content than did males (Table 9). This is probably the result of the lower MC numbers in females (Table 5) and the lower and/or more variable HA content of these cells in females (Results IV-B). That correlations of thalamic MCs versus thalamic HA content indicated a lower HA content in the brain MCs of females (Fig 17) than of males and that the significance of the correlations was slightly less in females than in males supports this argument.

Correlations of thalamic MCs and whole brain HA were highly significant in both sexes and indicated that, by whole brain analysis, thalamic MCs contained approximately 3 pg of HA per MC. Again, this value is much lower than that of MCs found in the periphery and is similar to the values found using thalamic analysis. Similar correlations using whole brain HA levels indicated that thalamic MCs can significantly contribute to the levels of HA in whole brain (Fig 20). The values of non-MC HA levels in whole brain (42 ng/g) are virtually the same as the whole brain HA values obtained by previous investigators (Green, 1970; Hough and Green, 1984).

3) Contribution of rat brain MCs to brain HA - the characterization of brain HA as a neurotransmitter has been hampered by the concept of a possible contribution of non-neuronal HA to overall brain HA pools, masking and clouding the ability to determine neuronal levels and turnover. Most authors have attributed any non-neuronal source of HA to only one cellular population, the brain MC. In order to characterize the contribution of brain MCs to brain HA, they have relied on essentially 2 experimental protocols: 1) anatomical quantification of brain MCs and estimation of their contribution based on HA values measured in other laboratories and with other animals and 2) biochemical characterization of "neuronal" HA and attribution of any "non-neuronal" HA to MCs. Both these methods have had major flaws in them that precluded unequivocal identification of either the MC pool of HA or its contribution. Further, many

of these studies were based on assumptions about the nature of MCs and neuronal HA that were not truly valid (see Introduction III).

The methods presented here allow for the determination of MC numbers and HA values from the same tissue, thereby taking into account individual variation in both MC numbers and HA. Thus, assumptions as to either the level or variability of either factor need not be made. However, this variability makes generalizations such as the overall population significance of a MC contribution to brain HA difficult to evaluate. Deviations from the ideal regression lines observed in the correlations of thalamic MC numbers with thalamic HA (Fig. 17) suggest that there are variations in either the HA content of the MCs (i.e., the slopes of the lines), or in the amount of non-MC HA present (the Y-intercept of the lines), or both. Thus, it is not possible from these data to determine the absolute percentage contribution of MCs to HA levels for a given thalamus. However, population approximations can be made. If rat thalami contain 6.84 and 8.51 ng of non-MC HA (the intercepts of Fig. 17, both sexes), then thalamic MCs contribute 81% (0-92%) and 54% (0-82%) of the HA in thalamus in males and females, respectively. MCs account for 71% of the variation in thalamic HA in males and 46% of the variation in females (r^2, Fig 17).

Although thalamic MC numbers do not influence non-thalamic HA, they clearly can affect whole brain HA content.

Thalamic MC numbers were significantly correlated with whole brain HA amounts and levels (see above). These findings indicate that thalamic MC numbers account for 35-39% of the variation in whole brain HA levels (r^2, Fig 20). Assuming a constant non-MC contribution to whole brain HA levels (41.8 and 42.8 ng/g, Fig 20), our results indicate that thalamic MCs contribute approximately 30% and 20% of whole brain HA levels in males and females, respectively (a range of 0-50% for both sexes). In other words, up to one-half of rat whole brain HA levels can be contributed by thalamic mast cells.

iv) Other possible sources of brain HA aside from MCs - as discussed above, while rat brain MCs are capable of contributing HA to thalamic areas, their localization to thalamus precludes their contributing HA to other brain regions. Therefore, the biochemical evidence (lesion, subcellular distributional and HD inhibition studies, Introduction section III) that indicates a non-neuronal pool of HA in non-thalamic areas points to the strong possibility of at least a third storage depot for this amine. One possibility is the NLM cell postulated by Ibrahim (1974). These cells were supposed to have a more ubiquitous distribution in rat brain than MCs. Whether these cells contain HA or really exist is unresolved. However, purification of brain tissue to yield microvessels has added another piece to the puzzle. Jarrott et al. (1979) found that purified bovine microvasculature contained HA in concentrations of up to 1000 ng/g from cortex and 300 ng/g

from hippocampus and striatum, a 6 to 23 fold increase in levels over the original homogenates (50 ng/g). This HA was associated with minimal HD activity and sedimented in the P_1 subcellular fraction, characteristics reminiscent of MC HA. Recovery calculations indicated that these microvessels were capable of contributing up to 50% of total cortical HA, a value similar to that obtained with biochemical characterization of neuronal HA (see Introduction section III). While histochemical analysis of this vasculature was not performed, electron dense granules of variable size were visualized in these vessels (Head et al., 1980). Whether these granules were from the NLM is not known, but this evidence certainly points to the possibility of other cellular elements in brain that might contain HA. It is unlikely that these populations are numerically related to MCs, as MC are not seen in cortex.

C) Thalamic MCs and brain tMH - this investigator attempted to determine whether thalamic tMH was related to thalamic MCs or was a function of some other parameter. Thalamic tMH levels and content (Table 10) were both lower and less variable than thalamic HA (Table 9). Thalamic tMH (content: approximately 6 ng; levels: approximately 76 ng/g) was similar to thalamic non-MC HA (content: approximately 6 ng; levels: approximately 100 ng/g; Figs 17,18) rather than total thalamic HA parameters. These levels were similar to those found by other investigators (Oishi et al., 1983: 57 ± 7 ng/g, CV%=24%; Oishi et al., 1984: 83 ± 8 ng/g, CV%=23%; Hough

and Domino, 1979: 50 ng/g). Thus, it is possible that thalamic tMH is a function of thalamic non-MC HA (i.e. thalamic neuronal HA) rather than being a function of thalamic MCs (see Introduction section IV). 1984a).

That thalamic tMH levels showed no relationship to thalamic MCs in males (Figs 21,22) increases the liklihood that thalamic tMH is due to the thalamic non-MC HA rather than thalamic MCs. In females, the situation was less clear. Although brain MCs failed to correlate with thalamic tMH levels (Fig 22), they did correlate significantly ($P=0.016$) with thalamic tMH content (Fig 21). The correlations of thalamic MC numbers with thalamic tMH content in males and females were not different by z transformation analysis and the common correlation was significant ($P<0.02$, Results V-A). The discrepant results of thalamic tMH content and levels relative to thalamic MC numbers is due to the variability in thalamic weight. These cells contain approximately 0.1 pg tMH per MC. This is 5% of the HA content of these cells.

D) Correlations of HA and tMH - thalamic tMH correlated significantly with thalamic HA in both males and females (Results section V-D). The question then remains as to whether this tMH is related to MCs or to non-MC HA. Partial stepwise correlation can determine the relationship between any two variables while holding other model variables constant (Zar, 1974). Performing such analysis helps to isolate relationships. Such analysis was performed on brain MCs, thalamic tMH and thalamic HA using the multiple

regression procedure on PROPHET. Brain MCs were significantly correlated with thalamic HA when tMH was held constant and thalamic HA was significantly correlated with thalamic tMH when brain MCs were kept constant (see Results V-D). However, when HA remained constant, brain MCs showed no relationship with tMH in either sex. Thus, thalamic tMH is a function of the non-MC HA present in thalamus rather than MCs. Therefore, the correlation between HA and tMH is accounted for by non-MC HA. This supports the hypothesis that brain tMH can be used as an indicator of histaminergic neuronal activity (see Introduction section IV).

E) Replicability of the MC versus HA correlations - in an effort to determine whether the presented data for the estimation of thalamic MC numbers and their correlation with thalamic HA was artifactual, a limited re-analysis was performed on 6 adult male rats (body weight: 296±5g, range=275 to 310g). Mean estimated MC numbers were 8060±1834 (range=2206 to 12802, CV%=56%) and were not different from the previous data set by group t-test. Thalamic HA values were significantly lower than those seen earlier (186±29 ng/g, 88 to 286 ng/g) but maintained their large variability relative to non-thalamic values (thalamic CV%=38%; non-thalamic CV%=16%). Non-thalamic HA levels were also slightly lower but not different from those seen previously (38±2.5 ng/g, range=30 to 47 ng/g) and whole brain HA levels were significantly lower (44±3 ng/g, range=35 to 53 ng/g, CV%=17%), reflective of the lower thalamic HA. Of major

interest was the finding that the correlation of thalamic MC numbers with thalamic HA content (ng) was close to being significant even for this low n value and limited range in MC numbers ($r=0.763$, $P=0.078$) and the fitted parameters (intercept=8.0 ng, slope=1.0 pg HA per MC) were similar to those seen previously. ANCOVAR of this and previous data for MCs versus thalamic HA indicated no difference in the slopes of the two lines. The intercept of the non-significant fitted line of thalamic MCs versus thalamic HA levels again indicated that thalamus contains non-MC HA levels of 100 ng/g. The lower thalamic and whole brain HA values are undoubtedly due to the lower MC numbers within this group of animals, and the correlational statistics, while not significant, were the same as those seen previously and would have been significant (at this r value) had the n value been 8 instead of 6. These data indicate that the earlier data for estimated MC numbers and correlations with HA are not artifactual, and, regardless of their overall validity towards estimation of the contribution of MCs to brain HA, can be replicated. Thus, our data are reflective of true population parameters. This series of experiments further underlines the cautions that must be taken when attempting to evaluate multiple experimental parameters that have large independent variabilities. The variables accounting for MC numbers are not understood, but must explain the discrepant findings.

V) Comparison of HA levels in tissue dissected while either fresh or frozen

As discussed (Results section V), the very high thalamic HA values seen in the MC-HA correlational study, the variability in these levels relative to non-thalamic regions, and the non-MC HA levels and contents in thalamic and whole brain regions, seemed to indicate that we were assaying a MC component of brain HA that had not been previously measured or had been overlooked. An evaluation of my methodology indicated that the major difference between this study and those previous was my practice of freezing brain tissue solid prior to dissection. I felt that this might be preserving some MC HA that might otherwise be lost or masked when dissections are performed on fresh tissue. This impression was bolstered by data that chilling brain tissue in liquid N_2 increased the HA levels in all regions examined, but that the effect of different temperatures of decapitation was the greatest for the diencephalon (Taylor and Snyder, 1971). Further, the group that found the highest and most variable thalamic HA levels (Oishi et al., 1983,1984) in previous reports (Table 1) chilled the decapitated heads of their animals in liquid N_2 prior to brain removal and dissection. I thus undertook a study to evaluate the effect of temperature of brain processing on the HA levels in different brain regions.

The underlying supposition of the experiment was that should freezing maintain or unmask a distinctly MC component

of brain HA, then any differences between fresh and frozen tissue HA content should be confined to thalamus. If this phenomenon were related to HA levels rather than MCs, then differences might be observed in hypothalamus as well. No differences were expected in brain areas that had lower HA levels and no or few MCs, i.e., cerebellum, basal ganglia and cortex. Towards this end, I isolated thalamus, hypothalamus, cortex and cerebellum from the brains of both experimental groups (dissected fresh or frozen). However, difficulties in dissecting frozen brain tissue constrained me to include brainstem tissue with the cerebellum and to group all of the remainder of the brain outside diencephalon with the cortex. The four groups so obtained were homogenized and analyzed for HA in an identical manner except for the state in which the brains were dissected and stored prior to homogenization.

Unexpectedly, there were no differences between fresh and frozen processed tissue in any region examined (Table 12, Fig 22). Hypothalamic, cerebellum/brainstem and cortex/remainder regions had HA levels similar to values published previously (Table 1). The variability in the HA levels of these regions was low, being similar both to previous studies and to the non-thalamic HA variability seen in the MC-HA correlational analysis presented above. Thalamic HA levels, though not different between fresh and frozen brains, and though lower than the high values seen previously (Table 9), were still higher than the values published earlier (except Oishi et al., 1984; Table 1). The thalamic HA levels maintained the

large variability seen earlier. These thalamic HA values were similar to those found in the smaller (n=6) MC-HA study discussed above (Discussion section IV-E).

The lower thalamic HA levels seen in this experiment relative to earlier results (Table 9) are most likely due to lower MC numbers being present in this group of animals. This statement is bolstered by the similar HA levels in the small (n=6) MC-HA analysis presented above.

VI) Peripheral administration of compound 48/80 and degranulation of rat brain MCs

A number of studies have given peripheral administration of the MC degranulating agent compound 48/80 in attempt to determine whether brain MCs were responsive to this drug or to isolate MCs from other cellular elements in brain. However, 48/80 is a polar molecule and there is no evidence that it penetrates the blood-brain barrier. Our findings that large doses of 48/80 had no degranulating effect on brain MCs yet were active on MCs in tongue argues against its ability to enter brain. There is no information concerning the half-life of this drug in blood.

Ibrahim (1970) gave rabbits and cats 48/80 i.p. over a two day period (2 mg/kg, 1x or 2x per day) and sacrificed the animals one to 21 days later. He observed the brain MCs showed signs of degranulation and there was a decrease in MC numbers. This phenomenon required two days before it could be observed. He later replicated this study (1974) and

indicated that rats were less sensitive to this agent than were rabbits and cats. Although he made no distinction between MC types in the 1970 paper, he later indicated that these cells were type II MCs (1974). Cammermeyer (1976) also gave 48/80 i.p. to cats (no dosages or protocol was described) and indicated that there may have been some degranulation of MCs in area postrema. However, MCs in habenular nuclei were resistant to the drug. As area postrema has a less intact blood-brain barrier than does thalamus, it seem likely that 48/80 is not capable of reaching thalamic MCs following acute administration.

Martres et al. (1975) injected one day old rats with 48/80 (1 dose, 20 mg/kg s.c.) and examined brain HA levels one hour later. There was no significant drop in whole brain HA relative to controls. No mention was made of the condition of the neonatal animals following the drug administration. However, dosages in excess of 5 mg/kg are lethal to adults (Results section II). Pollard et al. (1976) gave chronic 48/80 to adult rats (1 to 2.5 mg/kg per dose over a 3 day period) and observed a nearly 50% drop in HA in the median eminence. Other hypothalamic nuclei were not affected and other brain regions were not examined.

Persinger (1983) injected neonatal and young rats with 48/80 (IP) both acutely and chronically (over a five day period) and observed no morphological signs of degranulation in thalamic MCs. MCs in peripheral tissues were severly effected.

In summary, there is no strong evidence that 48/80 is capable of entering the brain and degranulating thalamic MCs following peripheral administration. Areas that have shown some indication of such behavior have a less intact blood-brain barrier than thalamus. Although it is possible that 48/80 penetrates the brain slowly and remains in the blood long enough to do so, no data supports such a hypothesis. An undeveloped blood-brain barrier in neonates might account for the findings of Martres et al. (1975).

VII) Rat brain MCs and possible functions in-vivo

While the presented data indicates that MCs are present in rat brain and that they contain at least one amine that is capable of profound vascular and/or neuronal activities, the question as to what role these cells might play in normal brain physiology remains unanswered. As discussed, some evidence has been presented indicating that MCs might be implicated in sensory processing. However, whether MCs played an active role or just responded secondarily to vascular or neuronal metabolic changes is not known. As the hypothesis that MCs might be a gateway between the neuronal, immunological and hormonal systems has been put forward (Persinger, 1977a; Goldschmidt et al., 1984b), some of the evidence supporting such a concept bears investigation.

A) MCs and direct neuronal interactions - while there is no evidence supporting a direct interaction between MCs and neurons in the CNS, circumstantial data support this

possibility. Such evidence takes two forms, analysis of MC surface receptors known to be capable of interacting with neurotransmitters found within the CNS and anatomical evidence linking MCs with neurons.

1) Studies showing MCs with direct contact to nerves - several studies using the electron microscope have found evidence linking MCs and neurons both in the peripheral and the CNS. Heine and Forster (1975) showed that MCs in dog cardiac tissue and human subcutis had direct contact with small unmyelinated preterminal nerve fibers. In some cases, fibers penetrated the MC cytoplasm. However, the pH of the toluidine blue used to visualize these MCs was high (pH 5), so that the identification of these cells as MCs was not complete. A similar relationship between unmyelinated nerve fibers and MCs was observed in a human subungual glomus tumor (Wiesner-Menzel et al., 1981). In this case, two classes of MCs were observed. One group, which was termed mature MCs due to numerous granules and few lamellipodia, was situated close to nerve bundles, in some cases as close as 20 nm. The second group, termed immature MCs due to their lower granular content and many lamellipodia, was always in contact with these nerves, sometimes with their protrusions surrounding the nerve fiber or even penetrating it. While these authors termed both groups of cells MCs, the pH of their stain was higher than it should have been (pH 4.1). Further, a well developed Golgi apparatus, mitochondria and numerous ribosomes might have indicated an activated MC rather than an

immature cell. That neurons might have induced MCs to activity would be excellent evidence for a role of MCs in rat brain. MMCs have also been demonstrated to have direct contact and synapses with sub-epithelial nerve bundles (Newson et al., 1983). Within brain, MCs of the hedgehog were observed to give off lamellipodia that were observed to have direct contact with myelinated nerves (Flood and Kruger, 1970). Thus, evidence suggests that MCs might be capable of communicating with neurons.

2) Neurotransmitter receptors on MCs - whether MCs posses the biochemical machinery for the chemical communication with neurons has been controversial for some time. Cholinergic muscarinic receptors have been reported to be present on rat peritoneal MCs (Masini et al., 1981), yet were not involved in HA release. An other study failed to find any such receptors on rat MCs (Donlon et al., 1982). Fantozzi et al. reported that cholinergic agents were capable of the non-cytotoxic degranulation of MCs at low concentrations (10^{-11} M; Fantozzi et al., 1978a,1979) and that the release was by muscarinic mechanisms (Blandina et al., 1980). While some authors have found evidence to support the concept that cholinergic mechanisms can result in MC activation and degranulation (Schmutzler et al., 1978b,1983) or modulation of function once stimulated (Erjavec and Iskra, 1984), others have failed to confirm these results and could find no effect of ACh or cholinergic agents on MC function (Kazimierczak et al., 1980; Foreman, 1981; Leung and Pearce, 1984). While the

cholinergic innervation of thalamic nuclei has not been completely established, ACh has been implicated as a neurotransmitter in the ventral lateral, reticular, anterior and ventral lateral geniculate nuclei (Jones, 1981,1983).

The role of adrenergic agents on MC function has been confusing. Beta-adrenergic binding sites have been assayed on rat MC membranes (Donlon et al., 1982; Marquardt et al., 1982). However, the activity of exogenous adrenergic agents has been controversial and follows no set pattern that has been able to be characterized as either alpha or beta. Fantozzi et al. found that adrenergic agents (noradrenalin, NA) inhibited cholinergic HA release through a beta-specific mechanism (Fantozzi et al., 1980) and increased spontaneous release (no dose response curves were shown) by an alpha-dependent mechanism (Fantozzi et al., 1978b). Mannaioni et al. (1975) reported that NA was capable of degranulating mouse neoplastic MCs at moderate concentrations (10^{-4} M), yet Johnson and Moran (1970) were unable to observe this phenomenon in rat MCs at concentrations up to 1 mM. Whether this was due to methodological or species differences or was due to the neoplastic nature of the mouse mast cells is unknown. Adrenergic agonists have been shown to be capable of inhibiting release from MCs activated by 48/80 or antigen, but this activity has been characterized both as beta (Johnson and Moran, 1970; Belcheva et al., 1984) and alpha (Alm and Bloom, 1981). The inhibitory effect of NA on 48/80 induced release was observed to be potentiated by beta-

blockers (Alm and Bloom, 1981), yet these blockers, by themselves, were shown to be capable of inhibiting the same release (Nosal and Menyhardtova, 1977). Thus, what activity NA might have on brain MCs is not known. The primary localization of NA innervation in rat thalamus is the paraventricular nucleus (Jones, 1983).

The ability of HA to modulate MC activity is much better established than for cholinergic or adrenergic mechanisms. It is now well known that HA is capable of the inhibition of MC degranulation at concentrations of approximately 10^{-6} M and that this inhibition is H_2 specific (Lichtenstein and Gillespie, 1973; Masini et al., 1982: see reviews by Holroyde et al., 1977; Plaut and Lichtenstein, 1982). Masini et al. (1982) have reported being able to measure high affinity H_2 (dissociation constant, K_d=1.8 nM) binding sites through the use of ^{3}H-cimetidine. However, the K_d of the H_2 receptor (i.e., the concentration required to occupy 50% of the receptors) is $6x10^{-7}$ M (see Hough and Green, 1984) and the authors did not use this drug at concentrations higher than 20 nM. This means that a) the K_d value for the H_2 receptor that was measured by Masini et al. (1982) was greater than two orders of magnitude lower than that observed by others, and b) unless they (Masini et al., 1982) were measuring a new HA receptor (which is unlikely), the concentrations of ^{3}H-cimetidine that was used would have been sufficient to occupy only a very small fraction of the total H_2 binding sites available on the cell. Thus, the findings of Masini et al.

(1982) are of doubtful validity. As the thalamus is sparsely innervated with histaminergic neurons, it is doubtful that this mechanism would be of significance in any neuronal-MC interaction.

The observation that rat peritoneal MCs seem to have an opiate receptor (Yamasaki et al., 1982) raises the possibility that brain MCs might interact with the opiate peptides found in brain. While enkephalin fibers have been inferred to be present in the paraventricular, ventral lateral geniculate and intralaminar nuclei, the paraventricular nucleus is the only area presentlyiknown to contain beta-endorphin reactive fibers (Jones, 1983).

B) Immunological capabilities of brain MCs - recent evidence has been published that indicates that brain MCs are capable of immunological function in-vivo. NC-mice, similar to rats, were shown to have brain MCs (characterized by acidified 1% toluidine blue pH 2.7) localized to thalamus, primarily in dorsal thalamus, and the nuclear distribution within thalamus was similar to that presented in this thesis (Table 6) (Ishibashi and Sawano, 1982). The numbers in control animals were found to be a mean of 449 (range=290 to 632), similar to that found by Hough et al. (1984b) and Orr and Pace (1984). Following peripheral sensitization of the animal with egg ovalbumin, MC numbers in thalamus were shown to increase to a mean of 1433 (range from 1320 to 1705). Further, the HA content of these cells, as measured by the intensity of fluorescence following gaseous OPT treatment,

increased in these cells following the sensitization procedure. Following challenge, MC numbers and HA fluorescence were observed to decrease, and morphological evidence showed signs of MC degranulation and low granule density. While this evidence is extremely exciting, no statistical data were presented, so no definitive statements can be made concerning this study. Further, brain MCs have been characterized as being inside the blood-brain barrier (at least with respect to polar drugs such as 48/80, see Results section I-D and Discussion section VI). Unless these NC-mice have a defective barrier in thalamus, it is difficult to understand how a large molecule such as the IgE antibody could cross the barrier and sensitize the MCs.

As discussed (Introduction section I-H-iii), MCs have been suggested to be an integral part of the DTH reaction in mice. A recent study (Mokhtarian and Griffin, 1984) has indicated that brain MCs may be capable of the same function in-vivo. The mast cell-deficient mouse (W/W^v) was analyzed for its ability to mount a DTH reaction relative to its +/+ control following innoculation with Sindbis virus, which causes a CNS-localized DTH inflammation. The mutant mice were shown to be significantly less capable (at certain time points) of mounting a response than were their control counterparts. Virus clearance titers and other immunological criteria were not different between the 2 groups, indicating that the depressed DTH response was due to a lack of MCs in the brains of W/W^v animals and was not secondary to a

generally depressed immune system. Major criticisms of this paper were that no attempt was made to visualize MCs histochemically and the results contained no statistical analysis. However, ample evidence exists that W/W^v animals have no brain MCs and that controls have low to moderate numbers (Hough et al., 1984b; Orr and Pace, 1984). How this study relates to evidence that peripheral DTH responses are unimpaired in W/W^v mice (Introduction section I-J) is not known.

These two papers are the first concrete evidence that brain MCs may function immunologically in-vivo. Whether these cells also function in other species is yet to be determined.

C) Human pathological syndromes in which brain MCs have been implicated - MCs have been reported to be present in the lesions and plaques of multiple sclerosis and syphilis (Olsson, 1968,1974). As the symptoms of these diseases are primarily inflammatory in nature, it is entirely possible that brain MCs function in a similar manner as that described in the artificial DTH syndrome discussed above.

In addition to the inflammatory diseases, MCs have been implicated in headache and migraine pathologies (Theoharides, 1983). However, as Theoharides relied on 5HT as a primary mediator in the vascular disorders accompanying migraines, and this substance is only found in rodent MCs (Benditt et al., 1963), the use of a rodent model system in this human pathology may not be appropriate. Other mediators may be

involved. MC numbers from temporal skin biopsies were observed be higher in patients suffering cluster headaches than in controls (Liberski and Prusinski, 1982). These cells were said be actively extruding granules and were seen to be clustered around nerves. However, the statistical analysis used (multiple chi-squared tests) was inappropriate (they should have used ANVAR) and proper controls were not performed (identical placement of biopsies, non-temporal skin biopsies, analysis of MC numbers from headache patients not in an active state). Further, HA release from other cellular elements has been implicated in this syndrome (Selmaj, 1983), leading to the question of whether MC function in headache (if any) might not be secondary to a more general phenomenon capable if affecting numerous cell systems.

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