PHARMACOLOGY OF CEREBRAL HISTAMINE

by

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Abstract

Four aspects of the function of histaminergic systems were studied in the rat brain: toxicology, catabolism, release in vivo, and high affinity binding of histamine. Preparations of histamine-N-methyltransferase (HNMT) derived from kidney and brain were employed in the radioenzymatic quantification of histamine in biological samples. Tritiated S-adenosyl-L-methionine ([3H]-SAM) served as the co-substrate.

A toxicological study was conducted to determine the sensitivity of the HA innervation to prenatal treatment with methylazoxymethanol (MAM), an inhibitor of mitosis. In adult rats, the MAM treatment was without effect on cerebral histamine content, although forebrain HNMT activity was 50% reduced. In another study, C-57 mice were treated with the selective dopamine neurotoxin l-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Substantial dopamine depletions were not associated with alterations in the cerebral histamine content.

In a study of the structural requirements for HNMT inhibition, 9-amino-1,2,3,4-tetrahydroacridine (THA), was found to be one of the most potent inhibitors yet described. The β-carboline alkaloids, of which harmaline is the prototype, were also found to be moderately potent HNMT inhibitors. Because of the lack of high-affinity re-uptake and the absence of alternate catabolic pathways, blockade of HNMT can potentially alter central histaminergic tone. Peripheral administration of THA was able to produce dose-dependent increases in cerebral histamine content, as was the more potent HNMT inhibitor, metoprine. The issue of structural requirements for HNMT inhibition are discussed in the light of these results.

The in vivo release of histamine was studied by the cerebral microdialysis technique. After chronic implantation of horizontal probes, TTX-insensitive and partially calcium-sensitive efflux of histamine was detected in the dorsal striatum and the bed nucleus of the stria terminalis. In striatum, histamine efflux was elevated 50% after peripheral histidine loading (500 mg/kg, i.p.). After synthesis blockade with α-fluoromethylhistidine (100 mg/kg,
i.p.), extracellular histamine levels in striatum disappeared in a bi-exponential manner. The half-lives of this disappearance, 32 minutes and 7 hours, indicate the presence of at least two histamine pools. Striatal histamine efflux was elevated by yohimbine treatment (10 mg/kg, i.p.), suggesting the presence of a tonic \( \alpha_2 \)-adrenergic inhibition of histamine release \textit{in vivo}.

In addition to the classical \( H_1 \) and \( H_2 \) receptors, histamine is able to bind to a pharmacologically distinct site, \( H_3 \), recently characterized as an autoreceptor regulating the synthesis and release of histamine. The binding properties of the \( H_3 \) ligand \( [^3H]\)-N\(^{\alpha}-\)methylhistamine (\( [^3H]\)-N-MeHA) were studied in forebrain cryostat sections by autoradiography. Determination of \( B_{\text{max}} \) (25 fmole/section) and displacement studies indicated that \( [^3H]\)-N-MeHA bound to the same site as \( [^3H]\)-histamine: the high affinity histamine binding site. Binding was greatest in the basal ganglia and had a complex distribution within the cerebral cortex. Quinolinic acid lesion studies indicated that the majority of the binding in the basal ganglia was on striato-nigral projection neurons. Cortical binding was also sensitive to local excitotoxic lesions. Therefore, the majority of \( H_3 \) binding is located on postsynaptic structures intrinsic to these brain regions, rather than on presynaptic autoreceptors on terminals of histamine neurons.
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I Introduction

A considerable body of evidence suggests a neurotransmitter role for histamine in the brain. [3H]-Histamine is synthesized in cortical slices from labelled histidine and may be released by depolarization (Verdiere et al., 1975). Reductions in the synthesis of histamine in forebrain regions following lesions to the medial forebrain bundle suggested the presence of a histaminergic projection ascending in that pathway (Garbarg et al., 1976). However, until the recent localization of histamine within specific neurons, histamine had remained a neurotransmitter without a home.

Even prior to the demonstration of neuronal localization, histamine had been implicated in the regulation of a wide variety of functions in the mammalian brain including arousal, water balance, body temperature and analgesia (see Hough, 1988). However, the functions of histamine in the nervous system are still poorly understood, so the present studies were carried out to characterize better some of the factors involved in the metabolism and functional aspects of histamine in the rat nervous system.

The pathway for synthesis and catabolism of histamine is illustrated in Figure 1. Histamine is formed from the essential amino acid histidine by the action of a specific enzyme, histidine decarboxylase (HDC, EC 4.1.1.22), which requires pyridoxal phosphate as the cofactor. HDC has a molecular weight of 54,000 when purified from fetal rat liver (Taguchi et al., 1984). A cDNA has recently been cloned, indicating a 73,450 molecular weight protein (Joseph et al., 1990). The discrepancy in the molecular weight may be related to post transcriptional or post translational modification of the gene product. The amino acid sequence derived from the cDNA is similar to that of other amino acid decarboxylases.

The mammalian HDC has a steep pH dependence curve: at pH 7, the optimum, the affinity for histidine was about 100 μM (Hakanson, 1967). Therefore, the enzyme is probably not saturated at normal physiological concentrations of histidine, which are on the order of 100 μM. Indeed, peripheral loading with large doses of the amino acid is able to increase
Figure 1. The Metabolism of Histamine

Histamine (HA) is formed from histidine by the action of histidine decarboxylase (HDC). In the periphery, HA may be deaminated by diamine oxidase (DAO), but in the central nervous system, catabolism involves the sequential action of two enzymes: histamine-N-methyltransferase (HNMT) and monoamine oxidase-B (MAO-B). The aldehyde intermediate formed by MAO is unstable and the carboxylic acid forms rapidly in the presence of oxygen. Histamine may act through at least three distinct receptor types.
L-histidine → HDC → histamine → HNMT → methylhistamine → MAO-B → methylimidazole acetic acid

imidazoleacetic acid → DAO → H1, H2, H3
cerebral histamine content (Schwartz et al., 1972). Several studies have indicated that HDC is regulated in an inhibitory manner by cAMP (Huszti and Magyar, 1984, Huszti and Magyar, 1985), although phosphorylation of the enzyme has not been formally demonstrated. However, histamine synthesis in slices is apparently not acutely regulated by alterations in HDC (Chudomelka and Murrin, 1989).

In the mouse kidney, HDC activity is induced by thyroxin and oestrogen and repressed by androgens. The mouse HDC gene seems to be associated with a testosterone-sensitive regulatory site (Middleton et al., 1987) responsible for the regulation of expression in kidney by steroid hormones. Castration results in two-fold increased brain levels of histamine (Orr and Quay, 1975), possibly related to elevated HDC activity. HDC and ornithine decarboxylase expression are induced by interleukin-1 and tumor necrosis factor in mouse peripheral tissues (Endo, 1989) but effects of these substances on cerebral HDC have not been studied.

The cerebral metabolism of histamine differs from that of other aromatic amine neurotransmitters in that a high affinity, saturable neuronal uptake mechanism appears to be absent (Schwartz et al., 1980, Smits et al., 1988), although some uptake may occur in glia (Rafalowska et al., 1987). Histamine-N-methyltransferase (HNMT, EC 2.1.1.8), a specific histamine methylating enzyme, was initially detected in mammalian brain (Brown et al., 1959). Brain is rich in HNMT, which specifically catalyzes methylation of the imidazole ring of histamine, with S-adenosyl-L-methionine (SAM) serving as the methyl-donating co-substrate. HNMT has since been purified from guinea pig brain and partially characterized (Matuszewska and Borchardt, 1983). The enzyme is highly specific for histamine: only histamine and a small number of side-chain modified compounds are substrates (Dent et al., 1982). The compartmental distribution of HNMT remains unclear: the enzyme is present in cultured glioma cells (Garbarg et al., 1975), but a substantial proportion of striatal HNMT is sensitive to kainic acid and electrolytic lesions of the medial forebrain bundle (Sperk et al., 1981), which is indicative of a neuronal localization.
Diamine oxidase, the enzyme mainly responsible for the catabolism of histamine in peripheral tissues, is virtually absent from the rat brain (Shaff and Beaven, 1976). Because of the absence of catabolic pathways other than N-methylation (Schwartz et al., 1971), and the lack of a robust histamine uptake mechanism, as described above, cerebral histamine content is potentially sensitive to HNMT inhibition.

The product of HNMT, tele-methylhistamine (τ-MeHA), is a substrate for monoamine oxidase-B (MAO-B) with a $K_m$ of 4 mM (Hough and Domino, 1978). The aldehyde formed by deamination is unstable and spontaneously oxidizes to τ-methylimidazoleacetic acid (τ-MeIAA) in the presence of oxygen. τ-MeIAA is presumably removed from the brain by an acidic metabolite transport system. As brain levels of τ-MeIAA are unaffected by probenecid treatment (Khandelwal et al., 1984), the metabolite is evidently removed by a mechanism other than the probenecid-sensitive acidic metabolite transport.

Inhibition of MAO-B with pargyline results in an accumulation of τ-MeHA in brain which is linear over a period of several hours (Hough et al., 1984). The accumulation of this metabolite has been used as a measure of histamine turnover. Regional turnover rates so calculated are altered by treatment with a variety of substances. For example, diazepam (Oishi et al., 1986) and barbiturates (Hough, 1987) substantially reduce the apparent turnover of cerebral histamine, effects which are presumably related to the depressant properties of drugs potentiating GABAergic transmission. Morphine treatment may either stimulate or inhibit the pargyline-induced accumulation of τ-MHA in different regions of mouse brain, an observation probably pertinent to the mechanism of the antinociceptive property of morphine (Licata et al., 1990).

The presence of histamine-containing neurons in the caudal hypothalamus of the rat has been demonstrated by immunohistochemical studies employing antibodies raised against purified HDC (Watanabe et al., 1983, 1984) or conjugates of histamine (Panula et al., 1984). In these immunohistochemical studies, histamine-containing neurons were localized in the tuberomammillary nucleus (TM), a magnocellular nucleus in the caudal hypothalamus. The
TM neurons have been shown also to contain a number of other neurochemical markers in addition to HDC, notably glutamic acid decarboxylase, which is generally a marker of GABAergic neurons (Vincent et al., 1983, Takeda et al., 1984), adenosine deaminase (Senba et al., 1985), and MAO-B (Vincent, 1989). A number of neuropeptides are found in the TM neurons, including galanin (Staines et al., 1986), TRH (Shirouzu et al., 1983), substance P (Kohler et al., 1985) and brain natriuretic peptide (Saper et al., 1989). The functional significance of the coexistence of so many substances remains to be determined.

In addition to the TM nucleus, histamine neurons have been observed in the horizontal layer of the guinea pig retina (Yamamoto et al., 1987), in several sympathetic ganglia in the rat (Häppölä, et al., 1985) and in neuronal elements of the gut (Ekblad et al., 1985).

The TM neurons provide a diffuse innervation of forebrain structures, including the cerebral cortex (Takeda et al., 1984, Watanabe et al., 1984), and striatum (Vincent et al., 1983, Steinbusch et al., 1986). A relatively dense innervation from the histaminergic TM neurons is present in the anterior hypothalamus of the rat (Staines et al., 1987) and specific structures such as the mesencephalic nucleus of the trigeminal nerve (Inagaki et al., 1987). Histamine fibres also project to the spinal cord (Wahlstedt et al., 1985), apparently without numerous axonal collateral projections to the forebrain (Takada et al., 1987).

Because of the technical problems related to the small volume of the TM nucleus, few afferents to the TM neurons have been clearly identified. Retrograde tracer studies have shown an innervation of the TM from the medial preoptic nucleus (Wouterlood and Gaykema, 1988), the diagonal band of Broca (Wouterlood et al., 1988), and the prefrontal cortex (Wouterlood et al., 1987). Immunelectron microscopic studies have demonstrated a number of synaptic inputs to TM neurons, including substance P (Tamiya et al., 1990), neuropeptide Y (Tamiya et al., 1989), and GABA (Ericson et al., 1989). The net effect of these identified inputs presumably contributes to the regulation of histaminergic tonus.

Histamine is able to interact with nervous tissue through at least three pharmacologically distinct binding sites in mammalian brain. Agonists of the $H_2$ receptor stimulate the
formation of cyclic AMP (Hegstrand et al., 1976) in a manner which is synergistic with the action of noradrenaline (Sek et al., 1988). In monkey cortex, the half-maximal stimulation of adenylate cyclase occurs at a histamine concentration of 10 μM (Newton et al., 1982). The histamine-sensitive adenylate cyclase is found in glia and the meninges as well as in neurons (Ebersolt et al., 1981). Activation of the H₁ receptor potentiates the effect of H₂ agonists on cyclic AMP formation in brain tissue (Palacios et al., 1978, Daly et al., 1980) and stimulates the hydrolysis of phosphatidylinositol (Fisher and Agranoff, 1987). A wide variety of antidepressant and antipsychotic drugs are able to block potently the histamine-stimulated formation of adenylate cyclase (Kanof and Greengard, 1978). Such effects are generally considered to be more closely related to side effects, rather than therapeutic actions, of these medications.

Given the close proximity of the TM nucleus to the median eminence and pituitary, it is not surprising that important aspects of the regulation of neuroendocrine function in rat brain should be regulated via histamine receptors. Basal and oestrogen-stimulated LH release (Miyaki et al., 1987) are increased by activation of H₁ receptors. In contrast, TSH release is inhibited by H₂ receptor activation (Di Renzo et al., 1987). The release of vasopressin is greatly enhanced by histamine (Dogterom et al., 1976), an effect mediated by both H₁ and H₂ receptors (Cacabelos et al., 1987). Likewise, basal prolactin release is stimulated by either H₁ or H₂ receptors, depending on the route of drug administration and the site of action (Knigge et al., 1988). The stress-induced release of prolactin is mediated by histamine, mostly via H₂ receptors (Knigge et al., 1988). Thus, H₁ and H₂ histamine receptors exert a variety of actions on the basal and evoked release of neurohormones.

The H₃ receptor, which is pharmacologically distinct from the classical H₁ and H₂ sites, has recently been described as a presynaptic receptor site which controls the synthesis and release of histamine, i.e. an autoreceptor (Arrang et al., 1983). The potassium evoked release of [³H]-histamine is an index of autoreceptor activation. In such experiments, N⁰-methylhistamine was characterized as a prototypic H₃ agonist (Arrang et al., 1983, van der
Wolf et al., 1987, Arrang et al., 1990) while thioperamide was described as an antagonist
(Arrang et al., 1987). If the $H_3$ binding site served principally as an autoreceptor, its
distribution in brain would be expected to correlate highly with the density of histaminergic
innervation. However, a single autoradiograph of $H_3$ binding (Arrang et al., 1987) suggests a
pattern of binding inconsistent with the innervation pattern.

Electrophysiological studies indicate that the post synaptic effect of histamine may be
either excitatory or inhibitory (Geller, 1980), with the predominant action being inhibitory in
cortical neurons (Haas and Wolf, 1977). Histamine is excitatory to supraoptic neurons via the
$H_1$ receptor (Armstrong and Sladek, 1985), but excitation of hippocampal pyramidal cells is
apparently produced via the $H_2$ receptor (Tagami et al., 1984). The latter excitation is based
upon the blockade of a calcium-dependent potassium current, which attenuates the
afterhyperpolarization (Haas, 1984). The electrophysiological effects of specific $H_3$ drugs have
not yet been characterized.
II: The Analysis of Histamine in Cerebral Extracts

(A) Introduction

The quantitative analysis of trace levels of histamine present in cerebral extracts may be considered a classical problem in neurochemistry. Histamine does not have a distinctive ultraviolet absorption spectrum, nor is it, in contrast to the catecholamines, highly active electrochemically. Histamine will react with formaldehyde to form a fluorescent species, but the spectral characteristics are not readily distinguished from those of the catecholamine-formaldehyde adducts, which are generally present in greater quantities. Most efforts to quantify histamine have relied upon derivatization procedures such as the o-phthaldialdehyde (OPA) method of Shore (1959). Histamine, when exposed to OPA at a basic pH, forms a fluorescent adduct. However, as OPA forms derivatives with many primary and secondary amines, it is important to avoid interference from compounds such as amino acids, catecholamines, and the polyamines, especially spermine and spermidine. Simple ion exchange purification of the extracts may remove the amino acids, but the highly basic polyamines are difficult to separate from histamine in a one-step procedure. Therefore, high performance liquid chromatography (HPLC) has been used to facilitate the analysis of histamine.

In HPLC assays of histamine, the derivatization may be performed either before or after the analytical separation. In the present study, initial efforts were directed towards replication of literature methods for the analysis of histamine in biological samples by HPLC with pre-column derivatization (Tsuruta et al, 1981, Skofitsch et al., 1981). Cerebral extracts were passed through a strong cation exchanger (Cellex-P), amines were eluted with hydrochloric acid and the samples were lyophilized. After addition of OPA solution to the dried extract, portions were analyzed by reverse phase HPLC with fluorescence detection. However, because of variation in the recovery of histamine, the results were too unreliable for routine analysis. The OPA-histamine adduct being difficult to separate from other mono- and polyamine
derivatives by means of HPLC, post-column derivatization methods have been developed (Yamatodani et al., 1985). Because equipment for the post-column method was not readily available, it was determined to employ a radioenzymatic assay for the analysis of histamine.

(B) Radioenzymatic Assay of Histamine and HNMT

HNMT, histamine and [³H]-labelled SAM ([³H]-SAM) may be used to form radio-labeled product, \( r \)-MeHA, which is readily separated from the methyl donor. As the enzyme recognizes only histamine and a few side-chain analogues (Dent et al., 1982), the specificity depends largely on the purity of the enzyme preparation.

In the general method for the HNMT assay, samples (5-100 \( \mu l \)) were incubated in 15 ml plastic tubes (Falcon) in the presence of an HNMT preparation, [³H]-SAM (New England Nuclear, 55-80 Ci/m mole), and 100 mM sodium phosphate buffer (pH 7.9). In some experiments, the specific activity of [³H]-SAM was reduced by the addition of various amounts of cold SAM (para-toluenesulfonate salt, Sigma). After incubation for between 15-60 minutes, reactions were stopped by the addition of 80 \( \mu l \) potassium borate (1 M, pH 10) followed by the addition of 4 ml toluene/isoamyl alcohol (3:1, BDH). The tubes were mixed on a vortex (20 sec). After a brief centrifugation, 3 ml of the organic phase was extracted with 300 \( \mu l \) of potassium phosphate buffer (1 M, pH 7.1) by mixing on a vortex (20 sec). The tubes were recentrifuged and the organic phase was removed by aspiration. The aqueous phase was re-extracted with 2 ml toluene/isoamyl alcohol (vortex, 10 sec), briefly centrifuged, and the organic phase was removed by aspiration. 250 \( \mu l \) of the remaining aqueous phase was placed in a 20 ml plastic vial to which was added 6 ml of scintillation counting cocktail (Amersham, Aqueous Counting Scintillant). In order to permit the extraction of the tritiated product out of the strongly buffered aqueous phase and into the organic counting phase, the scintillant had been modified by the addition of 1.2% v/v bis (2-ethylhexyl)-hydrogen
phosphate (Aldrich), an organic anion. Radioactivity was determined by five minutes counting in a Packard Tricarb 4530 liquid scintillation counter with the windows set for tritium.

In the present biochemical studies, four different HNMT preparations were utilized: a simple homogenate of rat brain (HNMT), an ammonium sulfate fraction from rat brain (HNMT₁), a DEAE purified fraction from porcine kidney (HNMT₂), and a highly purified commercial enzyme prepared from foetal rat kidney (HNMT₃). Sensitivity and conditions for linear assay were determined for each preparation. Protein contents of the various preparations were determined by the method of Lowry et al. (1951).

In some ex vivo experiments, HNMT activity was determined in crude homogenates from rat brain. Whole brain or specific regions were homogenized in four volumes of ice cold sodium phosphate buffer (100 mM, pH 7.9). 80 μl portions were incubated at 37 °C in the presence of 10 μM SAM (0.1 μCi) and 10 μM HA in a final volume of 150 μl buffer. The enzyme activity was calculated in units of nmole product/mg wet weight-hour.

HNMT₁ consisted of a crude ammonium sulfate fraction from whole rat brain (Brown et al., 1959, Taylor and Snyder, 1972). Whole rat brain was mechanically homogenized in 10 volumes of ice cold 0.25 M sucrose and centrifuged (37,000 x 60 min). From the supernatant, the 45-70% saturated ammonium sulfate fraction was taken. The final precipitate was dissolved in 2 ml phosphate buffer (10 mM, pH 7.4), dialyzed overnight at 4 °C in 4 l of buffer and stored at -20 °C. This preparation was stable for at least six months under these conditions.

HNMT₁ was employed for HNMT inhibition assays to be described in Chapter IV, where the kinetic properties of the enzyme are also described. Under standard assay conditions, a mixture consisting of 2.5 μl HNMT₁ (20 μg protein) and histamine (10 μM, Sigma) and 0.1 μCi ³H-SAM (10 μM) in a final volume of 150 μl sodium phosphate (100 μM, pH 7.9) were incubated at 37 °C. Under these conditions, the assay was linear for at least 30 minutes (Figure 2-A).
Figure 2. The Linearity of HNMT Assays

(A) HNMT₁, the rat brain fraction, produced a linear assay at times up to 30 minutes in the presence of 10 μM histamine and 10 μM SAM.

(B) HNMT₃, the commercial preparation, was linear up to 250 pg HA when incubated for 60 minutes at 4 °C, as suggested by the manufacturer. Standard assay conditions were as specified in the text.
**HNMT₁ Time Dependence**

- y-axis: nmol product/mg protein
- x-axis: Time (minutes)

**HNMT₃ Sensitivity**

- y-axis: Product (DPM x 10⁻⁴)
- x-axis: Histamine (pg)
HNMT\textsubscript{2}, which was used for the determination of histamine in tissue extracts, was prepared according to the initial steps of the method described by Harvima et al. (1985). Briefly, 100 g porcine kidney cortex were mechanically homogenized in 400 ml of ice-cold 30% sucrose. After centrifugation (37,000 g x 60 min), the 45-70% ammonium sulfate fraction was taken from the crude supernatant and, after overnight dialysis, purified by DEAE-sepharose ion exchange chromatography as follows: a DEAE column (20 x 1.5 cm) was equilibrated with 10 mM potassium phosphate (pH 7.4), 10% glycerol, 1 mM EDTA and 1 mM dithiothreitol (DTT, Sigma). After washing the column with 400 ml of the buffer, the kidney protein was eluted with a salt gradient (0-1 M NaCl) lasting eight hours at a flow rate of 67 ml/hour. The \( A_{280} \) of each 15 ml fraction was determined (Spectronic 20, Bausch & Lomb) and HNMT activity was estimated by incubation with HA (10 \( \mu \)M) and \( ^3 \)H-SAM (0.1 \( \mu \)Ci) for 30 minutes at room temperature. Results, as illustrated in Figure 3, indicate a substantial separation of HNMT from the main protein peak under these conditions. The DEAE peak fraction (65 ml) was concentrated on a 2 ml DEAE column and stored at -20 °C in the presence of 20% glycerol; HNMT activity was stable for at least six months. The activities of the HNMT\textsubscript{2} fractions, calculated in units of nmole product/hour, determined by incubation with 10 \( \mu \)M histamine and 0.1 \( \mu \)Ci \( ^3 \)H-SAM for 30 minutes at room temperature, indicated a 90-fold purification with an overall yield of 9% (Table 1). The HNMT\textsubscript{2} preparation was devoid of detectable catechol-O-methyltransferase and indole-methyltransferase activities.

The HNMT\textsubscript{2} assay was linear to 1000 pg HA (Figure 4-A). Addition of various amounts of external HA standard to the extracts likewise produced a linear assay (Figure 4-B). In routine assays, the HA was quantified by means of a two point standard curve with triplicate determinations of buffer and buffer plus 100 pg HA standard. In three separate experiments, the sensitivity of the HNMT\textsubscript{2} assay was 25 ± 5 pg HA as defined by twice blank and 7 ± 3 pg as defined by blank plus two standard deviations of the mean blank.

HNMT\textsubscript{3} was a commercial preparation (New England Nuclear), purified approximately 200-fold from foetal rat kidney by the method of Verburg et al. (1983). Standard assay
Crude ammonium sulfate fraction containing porcine kidney protein was eluted from a 25 cm DEAE column by a linear salt gradient to 1 M NaCl. HNMT activity (arbitrary units) and $A_{280}$ were determined for each fraction. The fractions between the bars were pooled and concentrated.
Table I. Purification of HNMT from Porcine Kidney

<table>
<thead>
<tr>
<th></th>
<th>Yield</th>
<th>Recovery</th>
<th>Activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant</td>
<td>807</td>
<td>100%</td>
<td>0.4</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>310</td>
<td>39%</td>
<td>0.25</td>
</tr>
<tr>
<td>Unconcentrated DEAE</td>
<td>78</td>
<td>10%</td>
<td>22</td>
</tr>
<tr>
<td>Concentrated DEAE</td>
<td>75</td>
<td>9%</td>
<td>35</td>
</tr>
</tbody>
</table>

The yield at each stage is reported in activity units of nmole product formed per hour under standard conditions. Activity is calculated from the yield in units of nmole product hour$^{-1}$ mg protein$^{-1}$.
Figure 4. Linearity of the Assay with HNMT$_2$

(A) HNMT$_2$ provided a linear assay for HA up to 1000 pg/sample.

(B) The addition of various amounts of external standard resulted in a linear increase in the HA content of rat brain extracts, as determined with the HNMT$_2$ preparation. Each determination performed in triplicate.
**HNMT₂ Sensitivity**

Product (DPM x 10⁻⁴) vs. Histamine (pg)

- Linear relationship
- Equation: \( y = 1.08(x) + 105 \)
- Correlation coefficient: \( r = 0.996 \)

Histamine Detected (ng/g) vs. Histamine Spike (ng/g)
conditions consisted of 25 μl sample, 2 μl enzyme, 25 μl buffer (100 mM, pH 7.9), and 1 μCi \(^{3}\text{H}\)-SAM. According to the suppliers, optimum sensitivity was obtained with 1 hour incubations at 4 °C. In three separate experiments the sensitivity of HNMT\(_{3}\) was 7 ± 2 pg/sample by the criterion of twice blank and 1.2 ± 0.3 pg/sample by the criterion of two standard deviations above the blank. The assay was linear to at least 200 pg HA (Figure 2B). The moderately greater sensitivity of HNMT\(_{3}\) with respect to HNMT\(_{2}\) indicated its use in the determination of the HA content of cerebral microdialysis samples.
III: Neurotoxicological Studies

(A) Introduction

Little is known about the sensitivity of the histamine neurons to neurotoxic challenge. If a selective neurotoxin were available, the biochemical and behavioral consequences of histaminergic deficiency could be better characterized. In the present study, attempts were made to interfere with histaminergic neurogenesis by exposure of foetal rats in utero to methylazoxymethanol (MAM), an inhibitor of mitosis (Nagati and Matsumoto, 1969). Another toxicological study concerned the sensitivity of the mouse histaminergic system to the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

i) MAM Methods

Because specific classes of neurons differentiate at different ontogenic stages, it is possible to block the development of specific lineages of neurons by administration of a mitotic inhibitor such as MAM. In the present study, precisely timed female Sprague-Dawley rats were administered MAM (25 mg/kg, i.p.) on G15 (Vincent et al., in press), while control dams received saline. Progeny were sacrificed at the age of six months. In six normal and six MAM-treated animals the regional content of histamine was determined in striatum, cortex, and hypothalamus and the HNMT activity was determined in neocortex.

ii) MPTP Methods

MPTP produces long lasting depletions of forebrain dopamine (Burns et al., 1983). The complete mechanism of toxicity remains to be elucidated, but an essential step involves the production of the metabolite N-methyl-4-phenylpyridinium ion (MPP⁺) by the action of
Table II. Effect of Prenatal MAM Exposure on Histamine Content and HNMT Activity in the Rat Brain

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Histamine (ng/g)</th>
<th>mg sample</th>
<th>ng total</th>
<th>HNMT Activity nmoles/mg/hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>33.9±2.5</td>
<td>63.7±1.2</td>
<td>2.15±0.14</td>
<td>1.68±0.06</td>
</tr>
<tr>
<td>MAM</td>
<td>44.1±1.8(^a)</td>
<td>36.5±2.1</td>
<td>1.6±0.08 (^a)</td>
<td>0.92±0.07 (^b)</td>
</tr>
<tr>
<td>(130%)</td>
<td>(57%)</td>
<td>(75%)</td>
<td>(57%)</td>
<td></td>
</tr>
<tr>
<td><strong>Striatum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>53.8±4.1</td>
<td>37.6±1.6</td>
<td>2.02±0.19</td>
<td>0.87±0.07</td>
</tr>
<tr>
<td>MAM</td>
<td>66.5±1.8(^a)</td>
<td>27.3±1.4 (^b)</td>
<td>1.82±0.11</td>
<td>0.58±0.05 (^b)</td>
</tr>
<tr>
<td>(124%)</td>
<td>(73%)</td>
<td>(90%)</td>
<td>(67%)</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>696±72</td>
<td>40±3.9</td>
<td>30.1±3.5</td>
<td>1.19±0.16</td>
</tr>
<tr>
<td>MAM</td>
<td>561±49</td>
<td>50.1±4.6</td>
<td>28.1±3.4</td>
<td>1.27±0.09</td>
</tr>
<tr>
<td>(81%)</td>
<td>(124%)</td>
<td>(93%)</td>
<td>(107%)</td>
<td></td>
</tr>
</tbody>
</table>

The histamine content in control (n=8) and MAM treated (n=6) animals is reported in ng/g wet weight. Values are mean ± S.E.M. of n determinations. The third column indicates the ng total histamine corrected for the weights of the samples. MAM values as a percentage of control values are indicated in parenthesis.

\(^a_p<0.025; \ ^b_p<0.001, \text{ Student's two-tailed t-test.}\)
MAO-B. The toxic metabolite subsequently accumulates within dopamine terminals via the high affinity dopamine uptake system (Sundstrom and Jonsson, 1986) and ultimately destroys these neurons. The site of generation of the MPP$^+$ relevent to neurotoxicity remains uncertain, but MAO-B dependant oxidation of MPTP has been demonstrated to occur in serotonin and histamine neurons (Vincent, 1989). Since the histamine neurons contain MAO-B and provide a widespread innervation throughout the brain, it is a possibility that they may participate in the production of MPP$^+$ within the basal ganglia.

In order to approach this issue, experiments were conducted to determine the sensitivity of the HA neurons to a dose of MPTP previously shown to deplete striatal dopamine (Radke et al., 1987). Young male C-57 mice weighing 25 g were given either 50 mg/kg MPTP hydrochloride (i.p., Research Biochemicals) or saline on two consecutive days and were decapitated two weeks after the last injection. Striatum, hypothalmus, neocortex and hippocampal samples were dissected, weighed and frozen at -70 °C until analysis.

Striatal dopamine (DA), serotonin (5-HT) and their metabolites were determined as described previously (Jakubovic et al., 1987). Briefly, tissues were extracted in 1 ml 0.2 M perchloric acid, centrifuged and analyzed by reverse-phase HPLC with electrochemical detection (Nucleosil C$_{18}$, 150 x 4.6 mm, Pharmacia). Histamine content of the other three tissues was determined using the HNMT$_2$ radioenzymatic assay.

(B) Results of Toxicological Studies

i) MAM Results

The regional histamine content and the cortical HNMT activity determined in the adult rat after MAM treatment at embryonic day 15 are indicated in Table II. Histamine concentration was elevated in forebrain structures, but these effects seemed to be due to reduced tissue volumes in forebrain structures of the adult MAM-treated brains. HNMT
activites were reduced in hippocampus (-45%) and striatum (-33%), but were not significantly affected in hypothalamus.

In tritiated thymidine studies, neurogenesis of HDC immunoreactive neurons occurred as early as embryonic day 13, but the peak of final mitosis is on day 16, which is rather late in gestation of the rat (Reiner et al., 1988). In the present study, MAM was administered on day 15, which should have killed any neuroblasts which would give rise to the histamine lineage dividing at that time. However, MAM-treatment kills actively dividing cells, not cells at other stages of the mitotic cycle. Therefore, the preservation of histamine content in the MAM rats suggests that a population of histamine neuroblasts was not dividing or was otherwise MAM-resistant on embryonic day 15. If HA neurons were reduced in number by prenatal MAM-treatment, the residual neurons were presumably able to provide a proportionally greater HA-innervation, perhaps in response to trophic factors in the forebrain. However, the effect of prenatal MAM on the number of HA neurons in the adult was not determined in the present study.

Interestingly, HNMT activity in the MAM-treated brain seemed to be more decreased by the MAM-treatment than were the histamine levels. As discussed above, the cellular localization of HNMT has not been well characterized. However, the MAM data suggest that a large proportion of HNMT activity in the adult forebrain appears to be derived from cells with final mitotis on or after embryonic day 15, the day of MAM administration.

ii) MPTP Results

The dose-dependent striatal dopamine depletions in mice receiving MPTP are illustrated in Figure 5 (Cumming et al., 1989). In the MPTP animals, DA declined to 28% of normal values and the concentrations of the dopamine metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT), were reduced to a lesser extent. 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were not significantly
Figure 5. Alterations in Dopamine and Metabolites After MPTP Lesions

The catecholamine and indoleamine content in mouse striatum, determined two weeks after saline (n = 8), or MPTP poisoning (n = 6), is represented as percentage of control values. Control values (ng/g) are: Dopamine: 11860 ± 290, DOPAC: 1860 ± 140 ng/g, HVA: 1543 ± 40, 3-MT: 877 ± 47, 5-HT: 408 ± 13, 5-HIAA: 426 ± 42. Results are indicated ± S.E.M.

(a) p<0.05, (b) p<0.001, Student's two-tailed t-test.
Table III. Effect of MPTP Treatment on Histamine Levels in the Mouse Brain.

<table>
<thead>
<tr>
<th></th>
<th>Hypothalmus</th>
<th>Hippocampus</th>
<th>Neocortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>155 ± 17</td>
<td>42.6 ± 3.8</td>
<td>29.9 ± 2.3</td>
</tr>
<tr>
<td>MPTP (110%)</td>
<td>170 ± 19</td>
<td>52.7 ± 7.7</td>
<td>28.8 ± 2.7</td>
</tr>
<tr>
<td>MPTP (124%)</td>
<td></td>
<td></td>
<td>(96%)</td>
</tr>
</tbody>
</table>

Histamine (ng/g) in three brain regions two weeks following two daily injections of either saline (n = 8) or MPTP (2 x 50 mg/kg i.p., n = 6). Results are reported as ng/g wet weight ± S.E.M. MPTP values are also reported as a percentage of control values.
altered by the treatment. Histamine levels, also, were not significantly affected by MPTP treatment (Table III), although there was a trend towards elevated concentrations in the hippocampus.

Concentrations of striatal dopamine, serotonin and metabolites in normal animals and the dopamine depletions produced in MPTP animals are similar to those reported in an earlier study (Radke et al., 1987) in which striatal noradrenaline was also determined and found to be 50% decreased in MPTP animals. However, in the present study, cerebral levels of histamine and the indoleamines were unaffected by MPTP treatment. The selectivity of MPTP for some of the ascending aminergic projections may be relevant to the relationship between the MPTP model and idiopathic Parkinson's disease, although it must be noted that C-57 mice are much less sensitive to MPTP than are the primates.
IV: Inhibition of HNMT

(A) Introduction

Because of the lack of high affinity uptake mechanisms or alternate catabolic routes, the major route for inactivation of cerebral histamine appears to involve the enzyme HNMT. Therefore, this enzyme serves as a metabolic bottleneck through which all cerebral histamine must pass. Thus, inhibitors of HNMT, of which there are many, have the potential to alter histamine levels. Indeed, metoprine, a diaminopyridine which potently inhibits HNMT, produces a long-lasting elevation of rat brain histamine levels (Duch et al., 1978). Lengthy lists of HNMT inhibitors have been compiled and attempts have been made to account for inhibition in terms of common structural features (Tachibani et al., 1988, Harle and Baldo, 1988). However, the diversity of known HNMT inhibitors suggests that the binding site or sites for substrates and inhibitors may have complex properties.

9-Amino-1,2,3,4-tetrahydroacridine (THA) is structurally similar to the quinacrine-derived antimalarial alkaloids, which are potent inhibitors of HNMT (Harle and Baldo, 1988). THA has been long known to be an acetylcholinesterase inhibitor (Kaul, 1962), which was the rationale for its experimental use as a therapeutic drug in Alzheimer's disease (Summers et al., 1986). Inhibition of HNMT by molecules with an aminoacridine nucleus has been previously noted, but not characterized (Duch et al., 1979).

A radioenzymatic assay was employed to study the sensitivity of rat brain HNMT activity to inhibition by THA, which was found to be one of the most potent HNMT inhibitors yet described. This inhibition was compared to that produced by various structural fragments and analogues of THA as well as other compounds, including metoprine, a potent HNMT inhibitor (Duch et al., 1980), and physostigmine, a classical acetylcholinesterase inhibitor.
Based on the results of the study of the structural requirements for HNMT inhibition, a number of \( \beta \)-carboline alkaloids were screened for their ability to inhibit HNMT. The \( \beta \)-carbolines, of which harmaline is a prototype, were found to be a new class of moderately potent HNMT inhibitors.

Subsequent to the characterization \textit{in vitro} of the inhibition of HNMT by THA, the degree of HNMT inhibition produced in rat cerebral cortex was determined \textit{ex vivo} at two hours after the peripheral administration of metoprine or THA. The effects of blockade of cerebral HNMT with THA or metoprine administration on the histamine content of cortex, striatum and hypothalamus were determined.

\textbf{(B) Materials and Methods}

The following compounds were employed in HNMT inhibition studies: metoprine (Burroughs-Wellcome, prepared in 5% lactic acid), physostigmine HCl, (Aldrich), 9-amino-1,2,3,4-tetrahydroacridine hydrochloride (THA, Sigma), 9-aminacridine hydrochloride (Fluka), 4-aminopyridine (4-AP) and 3-aminopyridine (Sigma), quinidine, 4-aminoquinaldine, 6-aminquinoline, 8-aminquinoline, and 8-amino-tetrahydroquinoline (Aldrich). The \( \beta \)-carboline compounds, harmalol hydrochloride dihydrate (4,9-dihydro-1-methyl-3H-pyrido[3,4-b]indole), harmol (1-methyl-9H-pyrido[3,4-b]indole), harmine hydrochloride hydrate (7-methoxy-1-methyl-9H-pyrido[3,4-b]indole), harmaline (4,9-dihydro-7-methoxy-1-methyl-3H-pyrido[3,4-b]indole), harmane (1-methyl-9H-pyrido[3,4-b]indole, aricine), norharman (9H-pyrido[3,4-b]indole) and 3-amino-1-methyl-5H-pyrido[3,4-b]indole were from Aldrich.

Inhibition studies were conducted using rat brain HNMT\(_1\) under standard conditions as described in Chapter II. HNMT\(_1\) activity was determined in the absence of inhibitors and at various final inhibitor concentrations ranging between 10\(^{-3}\) and 10\(^{-9}\) M. Inhibitor concentrations for 50% inhibition (IC\(_{50}\)) were determined by interpolation of the regression lines of the logit-log inhibition plots.
Kinetic parameters for the inhibition by THA were determined by Lineweaver-Burke analysis (Lineweaver and Burke, 1934). Rat brain HNMT\textsubscript{1} was employed under standard assay conditions, with one substrate at a fixed final concentration of 6.7 \( \mu \text{M} \) and the other substrate at five concentrations between 1-10 \( \mu \text{M} \). In the kinetic studies, THA was included at four different concentrations between 50 and 200 nM, while harmaline was included at three concentrations between 0.5 and 1.5 \( \mu \text{M} \). \( K_i \)s were calculated from the apparent \( K_m \)s in the presence of the various inhibitor concentrations according to the expression \( K_m[I]/K_m = (1 + [I]/K_i) \) where \( K_m[I] \) is the apparent \( K_m \) in the presence of the inhibitor (Gutfreund, 1965). Replotting the data by the method of Dixon yielded almost identical results (Dixon, 1953). Each assay was conducted in triplicate. Statistics, where reported, are \( \pm \) S.E.M. of \( n \) determinations.

For \textit{in vivo} experiments, groups of five male Long-Evans rats weighing between 200 and 250 grams were administered either saline, THA (5 and 10 mg/kg), or metoprine (10 mg/kg) by the intraperitoneal route. The rats were killed two hours later by cervical dislocation and samples of neocortex, striatum and hypothalamus were dissected, weighed and stored at -70 °C until analysis, no more than two weeks later.

HNMT activity in cortex samples was calculated as nmole product formed/minute-20 mg tissue and reported as the mean \( \pm \) S.E.M. of five determinations. Activity in samples containing HNMT inhibitors was also calculated as a percentage of the mean activity in control cortex samples.

Concentrations of histamine in cortex, striatum and hypothalamus after administration of THA or metoprine were determined employing a radioenzymatic assay using HNMT\textsubscript{2} as described above (Cumming et al., 1989). It was necessary to determine the linearity of the assay in the presence of known HNMT inhibitors. Therefore, between 0 and 250 pg of HA standard was added to portions of the pooled extracts of two whole rat brains. Two hours prior to sacrifice, the rats had been treated with either saline (1 ml/kg, i.p.), THA (10 mg/kg, i.p.) or metoprine (10 mg/kg, i.p.). The histamine assay was found to be linear both in the
Figure 6. Linearity of the HNMT\textsubscript{2} Assay in Presence of Inhibitors.

HA was determined in brain extracts to which had been added various amounts of exogenous histamine. Extracts were derived from animals which, two hours earlier, had received either saline, THA (10 mg/kg) or metoprine (10 mg/kg). Results are ± S.E.M. of four determinations.
presence and absence of inhibitors (Figure 6). The slopes of the standard curves determined in the presence of inhibitors were considerably reduced with respect to the slope of the assay of histamine standards alone, but the assay still had sufficient sensitivity to permit quantification of the histamine present in the extracts.

In the study of the effect of THA and metoprine on brain HA levels, the apparent histamine content of each extract was determined. Each sample was re-analyzed after the addition of 100 pg external standard HA. The recovery of the standard was quantitative in the case of samples from control animals and ranged from 30-75% in the samples containing the HNMT inhibitors. Linearity of the assay under these conditions having been demonstrated, histamine concentrations were corrected for individual standard recoveries and reported as ng/g.

(C) Results of HNMT Inhibition in vitro

The inhibition of HNMT by metoprine, THA, physostigmine and 4-AP are illustrated in Figure 7. Metoprine was the most potent inhibitor tested, with an IC\textsubscript{50} of 56 nM, while THA had an IC\textsubscript{50} of 74 nM. Physostigmine inhibited HNMT with an IC\textsubscript{50} of 8 \mu M and 4-AP with an IC\textsubscript{50} of 28 \mu M.

The log-logit analysis of the inhibition of HNMT by THA and eight related compounds is illustrated in Figure 8. IC\textsubscript{50}s were determined by interpolation of the regression lines. THA was the most potent inhibitor in the series, with an IC\textsubscript{50} of 130 nM, which is similar to the value determined from Figure 7. The THA fragment 4-aminoquinaldine, (IC\textsubscript{50} = 790 nM) was five-fold less potent than THA itself and 9-aminoacridine (IC\textsubscript{50} = 2.8 \mu M) was 20 fold less potent than THA. Quinidine, a quinoline alkaloid structurally related to quinacrine and quinine, was a moderately potent inhibitor, with an IC\textsubscript{50} of 8 \mu M. 4-Aminopyridine (IC\textsubscript{50} = 25 \mu M) was nearly 100 fold more potent than was 3-aminopyridine (IC\textsubscript{50} of 1.9 mM). The
Figure 7. Inhibition of HNMT by some compounds.

Inhibition of HNMT by, from left to right, metoprine, THA, physostigmine and 4-aminopyridine. Each point represents the mean of at least three determinations. S.E.M.s, omitted for clarity, were always less than five percentage units. Enzyme activity in the absence of inhibitors ($V_0$) was 150 pmole/min/mg protein.
Figure 8. Logit-log analysis of the inhibition of rat brain HNMT by THA and related compounds.

Rat brain HNMT was incubated in the presence of histamine (6.7 mM), $[^3]$H]-SAM (10 mM) and various concentrations of the following inhibitors: (a) THA, $IC_{50} = 130$ nM, (b) 4-aminoquinaldine, $IC_{50} = 790$ nM, (c) 9-aminoacridine, $IC_{50} = 2.8$ µM, (d) quinidine, $IC_{50} = 8$ µM, (e) 4-aminopyridine, $IC_{50} = 24$ µM, (f) 6-aminoquinoline, $IC_{50} = 540$ mM, (g) 8-aminoquinoline, $IC_{50} = 890$ mM, (h) 8-amino-tetrahydroquinoline, $IC_{50} = 1.3$ mM, and (i) 3-aminopyridine, $IC_{50} = 1.9$ mM. Enzyme activity in the absence of inhibitors ($V_0$) was 100 pmole/min-mg protein. $IC_{50}$ values were estimated by interpolation of the linear regression lines.
three aminoquinolines studied, in which the amine was in the 6- or 8- positions, were all poor inhibitors, with IC₅₀'s in the mM range.

HNMT inhibition plots for different concentrations of some β-carbolines are illustrated in Figure 9. Harmine (IC₅₀ = 1.9 μM) and harmaline (IC₅₀ = 4.4 μM) differ only with respect to the partial saturation in the 3-4 position. The saturation is evidently without great influence on HNMT inhibition. Likewise, harmol (IC₅₀ = 2.4 μM) and harmalol (IC₅₀ = 1.7 μM) were nearly equipotent HNMT inhibitors. The similar results obtained for the above four molecules indicate that the presence of the 7-methoxy group does not contribute to HNMT inhibition. However, harmane (IC₅₀ = 7 μM), which is unsubstituted in the 7-position, seems to be a somewhat poorer inhibitor than the others and norharman (IC₅₀ = 23 μM), which lacks the 1-methyl group, was poorer still. The least effective inhibitor in the series was 3-amino-1-methyl-5H-pyrido-[3,4-b]indole (IC₅₀ = 41 μM), which is actually not a β-carboline as it lacks a ring nitrogen in the 2-position. Among the β-carbolines, neither substituents in the 7 position nor the partial saturation of the first ring greatly change the HNMT inhibition. However, the 1-methyl group and the orientation of the aromatic nitrogens seem to influence potency as HNMT inhibitors.

The Kₘ of the rat brain enzyme preparation for HA was 3.3 ± 0.3 μM (Figures 10-A, 11-A) and the Kₘ for SAM was 4.3 ± 1.0 μM (Figures 10-B, 11B). These data are the mean of, respectively, three and four determinations, of which two are illustrated. The Vₘₐₓ was 197 ± 4 pmoles/minute-mg protein. As HNMT was subject to substrate inhibition at histamine concentrations above 10 μM, all assays were conducted in the appropriate range of concentrations. The above kinetic properties are comparable to those reported for purified guinea pig brain HNMT (Matuszewska et al., 1983).

THA inhibited HNMT in a manner which was competitive with respect to histamine (Figure 10-A, Kᵢ = 35 ± 6 nM, n=3) and displayed mixed competition with respect to SAM (Figure 10-B, Kᵢ = 39 ± 5 nM, n=4). The results of the kinetic study of the inhibition of HNMT by harmaline indicate that harmaline is a competitive inhibitor with respect to HA
(Figure 11–A, 1.4 ± 0.4 μM, n=3) and also SAM (Figure 11–B, 1.4 ± 0.2 μM, n=3). Analysis of all these data by the method of Dixon yielded nearly identical results.

The structures of some of the HNMT inhibitors used in the present study are illustrated in Figures 7 and 9. S-Adenosyl-L-homocysteine is reported to be a potent HNMT inhibitor, but the corresponding deaminated compound S-inosyl-homocysteine is a poor inhibitor (Zappia et al., 1969). This illustrates the importance of the 6-amino group on the basic moiety for cosubstrate binding (Figure 9, inset). HNMT inhibition is insensitive to the configuration of the asymmetric carbon in S-adenosylhomocysteine, while other methyltransferases were much less inhibited by D-analogs (Borchardt and Wu, 1974). These findings are further indication of the importance of the basic portion of SAM for binding to HNMT.

Examination of some of the structures in Figures 7 and 8 suggests that THA is an HNMT inhibitor by virtue of the 4-aminoquinoline structure, which may be superimposed on the 6-membered ring of the adenine moiety of SAM (Figure 9, inset). In contrast, 6- and 8-aminoquinolines were poor inhibitors, as was 3-aminopyridine. It is apparent that the integrity of the ring system is necessary for full HNMT inhibition by molecules in the THA series. As 9-aminoacridine was much less potent than THA, it appears that the degree of saturation of the acridine ring system can greatly alter the IC₅₀ for HNMT. The aminopyridine and aminoquinoline results indicate the importance of the orientation of the amino group with respect to the aromatic ring: only 4-amino compounds were good inhibitors. However, 4-aminopyridine was not a very good HNMT inhibitor. The importance of the complete THA ring structure for full inhibition suggests that hydrophobic interactions are involved in inhibitor binding. The competitive inhibition of HNMT by harmaline (Figure 11), which also may be superimposed on the basic portion of SAM and on a conformation of histamine (Figure 9, inset), suggests that the β-carbolines may directly interfere with the binding of both HNMT substrates.
Figure 9. Logit-log analysis of the inhibition of rat brain HNMT by some β-carbolines.

Rat brain HNMT was incubated in the presence of histamine (6.7 μM), [3H]-SAM (10 μM) and various concentrations of the following inhibitors: (a) harmalol, IC50 = 1.7 μM, (b) harmol, IC50 = 2.4 μM, (c) harmine, IC50 = 1.9 μM, (d) harmaline, IC50 = 4.4 μM, (e) harmane, IC50 = 7.0 μM, (f) norharmane, IC50 = 23 μM, and (g) 3-amino-1-methyl-(pyrido)indole, IC50 = 41 μM. Enzyme activity in the absence of inhibitors (V0) was 100 pmole/min/mg protein. IC50 values were determined by interpolation of the linear regression lines. Inset: structures of histamine and the adenine moiety of SAM are illustrated.
Figure 10. Double reciprocal plots of the inhibition of HNMT by THA

(A) Double reciprocal plots of the inhibition of HNMT by 55 nM and 110 nM THA with [³H]-S-adenosylmethionine held at 10 μM and histamine varying between 1 and 10 μM. Each point represents the mean of three determinations which varied by less than five percent.

(B) Double reciprocal plots of the inhibition of HNMT by 100 nM and 200 nM THA with histamine held at 6.7 μM and [³H]-S-adenosylmethionine varying between 1 and 10 μM. Each point represents the mean of three determinations which varied by less than five percent.
Figure 11. Double reciprocal plots of the inhibition of rat brain HNMT by harmaline.

(A) The concentration of histamine was varied between 1-10 μM and [3H]-SAM held at 10 μM. Harmaline was included at 0, 0.75, and 1.0 μM in this experiment. In a separate experiment, inhibition was determined in the presence of 1.5 μM harmaline. The structure of harmaline is illustrated.

(B) The concentration of [3H]-SAM was varied between 1-10 μM and histamine held at 6.7 μM. Harmaline was included at 0, 0.75 and 1.0 and 1.5 μM.
(D) Results of HNMT inhibition in vivo

The *ex vivo* inhibition of cortical HNMT activity by THA and metoprine is illustrated in Table IV. THA produced a dose-dependent inhibition of cortical HNMT. Metoprine was more potent than THA, producing nearly complete inhibition. This could be due to its slightly greater intrinsic potency or to greater penetration into brain tissue. The concentrations of THA and metoprine present in the *in vitro* assay following *in vivo* administration could be estimated by interpolation of the percentage inhibition from the inhibition curves for rat brain HNMT (Figure 7). Given a ten-fold dilution of inhibitors in cortex under the conditions of the assay, an approximation of the inhibition *in vivo* could be made by interpolation one log_{10} unit to the right on the inhibition curves. This provides only an estimation of the inhibition *in vivo*, but it is apparent that substantial inhibition of HNMT in cerebral cortex occurs at two hours after i.p. administration of moderate doses of the HNMT inhibitors.

The results of the determination of histamine concentrations in brain tissues after peripheral administration of HNMT inhibitors are illustrated in Figure 12. Histamine concentrations for control rat brain areas were similar to those reported previously (Oishi et al., 1984). THA produced a dose-dependent, approximately two-fold increase in cortical histamine. Metoprine produced a nearly three-fold increase in cortical histamine, consistent with its greater potency as an HNMT inhibitor. The drugs also elevated histamine in striatum, but the magnitude of the increases were lower than in cortex. In hypothalamus, a trend towards increased histamine levels did not reach significance in the THA groups and was marginally significant in the metoprine group.

The ability of a compound to inhibit HNMT *in vitro* does not necessarily indicate that it will discernibly influence histamine metabolism *in vivo* (Hough et al., 1988). The above data indicate that THA, as well as metoprine, are able to inhibit cerebral HNMT and greatly increase cerebral histamine levels at two hours after a peripheral dose. Given that THA is a lipophilic molecule, it seems likely that repeated administration of moderate doses of THA, as
has been proposed in the experimental treatment of Alzheimer's disease (Summers et al., 1986), could produce a cumulative inhibition of HNMT in the central nervous system.
Table IV. Inhibition of Cortical HNMT Activity

<table>
<thead>
<tr>
<th>Activity</th>
<th>% Inhibition in vitro</th>
<th>% Inhibition in vivo(^{(a)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.5 ± 0.9</td>
<td>-</td>
</tr>
<tr>
<td>THA 5 mg/kg</td>
<td>8.9 ± 0.4(^{b})</td>
<td>39%</td>
</tr>
<tr>
<td>THA 10 mg/kg</td>
<td>6.4 ± 0.8(^{b})</td>
<td>56%</td>
</tr>
<tr>
<td>Metoprine 10 mg/kg</td>
<td>1.9 ± 0.2(^{b})</td>
<td>87%</td>
</tr>
</tbody>
</table>

Two hours after i.p. drug injections, HNMT activity was determined in cortical homogenates. Values are in units of nmole/min-20 mg ± S.E.M. (n=5). Inhibition \textit{in vitro} is also reported as a percentage of control activity. (a) Inhibition \textit{in vivo} was estimated from the inhibition curves in Figure 7 as follows: To correct for ten-fold dilution of the inhibitors during the assay, the concentrations of inhibitors \textit{in vitro} were estimated by interpolation of the inhibition curves. The \textit{in vivo} inhibitions were taken from interpolations of the curves at concentrations one log unit to the right.

\(^{b}\)P<0.001, Student's two-tailed t-test.
Figure 12. HNMT inhibitors elevate cerebral histamine levels

Animals were sacrificed at two hours after saline, THA (5 and 10 mg/kg) or metoprine (10mg/kg). Histamine content in brain tissues from control animals were: neocortex (36.7 ± 1.0 ng/g), striatum (36.5 ± 1.6 ng/g), and hypothalamus (349 ± 25 ng/g).

(a) P<0.1, (b) P<0.025, (c) P<0.001 Student's two-tailed t-test.
Histamine Levels at Two Hours
After i.p. HNMT Inhibitors

C = control
1 = THA, 5 mg/kg
2 = THA, 10 mg/kg
3 = metoprine, 10 mg/kg
V: Cerebral Microdialysis

(A) Introduction

Brain histamine metabolism has generally been studied in ex vivo experiments employing tissue extracts and slice preparations. The synthesis and release of $[^3]$H-histamine by brain slices has been shown to be under the regulation of a variety of factors including histamine H$_3$ autoreceptors (Arrang et al., 1987), and $\alpha_2$-adrenergic receptors (Gulat-Marnay, 1989). In vivo release of histamine has been demonstrated by means of push-pull cannulae in the hypothalamus of conscious rabbits and anesthetized cats (Philippu et al., 1982, Prast et al., 1989). The study of cerebral histamine by microdialysis has recently been reported in acute anesthetized preparations (Yamatodani et al., 1989, Russell et al., 1990).

In the present study, cerebral microdialysis was employed in order to characterize some of the factors regulating the extracellular levels of histamine in the brain of freely-moving, unanesthetized rats. To this end, a sensitive radioenzymatic assay was employed in order to quantify histamine in cerebral dialysate samples. The dorsal striatum was chosen as the principle target of these studies because it receives innervation from the tuberomammillary nucleus (Steinbusch et al., 1986, Vincent et al., 1983), and because of possible interactions between histamine and psychomotor behavior (White and Rumbold, 1988). Some studies were conducted in the bed nucleus of the stria terminalis (BNST) because this region contains one of the highest histidine decarboxylase (HDC) activities in the rat nervous system (Ben-Ari et al., 1977).

(B) Materials and Methods

The protocol for cerebral microdialysis was based on methods described previously for the study of dopamine and its metabolites (Imperato and DiChiara, 1984, Westerink and
Tuinte, 1986) Briefly, male rats of the Wistar strain (200-300 g) were anesthetized with sodium pentobarbital (70 mg/kg i.p.) and placed in the stereotaxic apparatus (Kopf). After exposure of the skull, holes were drilled in the temporal bones so as to permit the placement of a horizontal dialysis probe. Two brain areas were targeted in this study: dorsal striatum (V: -4.75, A: +0.7) and the BNST (V:-6.60, A:-0.5) according to the coordinates of Paxinos and Watson (1986). Each dialysis fibre, consisting of saponified cellulose ester (o.d. = 0.27 mm, 10 kD cutoff, Cordis Dow Medical), was covered with glue except for two 3.5 mm lengths for the dorsal striata and two 1.7 mm lengths for the BNST. Stainless steel cannulae, glued to both ends of the dialysis fibre, were secured with dental cement to three anchoring screws on the top of the skull.

After surgery, rats were singly housed in cubical Perspex cages (40 cm) and allowed free access to food and water. Microdialysis perfusion experiments were carried out 24-72 hours after surgery. Experiments began at 11 A.M. and lasted no more that five hours, during which time the rats were unrestrained and allowed free access to food and water. The steel cannulae were connected to polyethylene tubing (i.d. = 0.28 mm, length = 80 cm) and the dialysis fibres were perfused with a solution containing 147 mM NaCl, 3 mM KCl, 1.3 mM CaCl₂, 1.0 mM MgCl₂ and 1.0 mM sodium phosphate (pH 7.4) at a rate of either 3 or 5 μl/min. Samples were collected for 20 minute periods in Eppendorf tubes and frozen at -20 °C until the day of analysis. Some brains were taken for histological verification of the membrane placement.

In the cerebral microdialysis study, the highly purified HNMT₃ was used because of its greater sensitivity. The histamine content in 25 μl volumes was determined and calculated as pg/minute sample. Because of the nature of a radioenzymatic assay, the possibility of interference from inhibitors must always be considered. Therefore, in experiments where the composition of the perfusion solution was changed, linearity of the assay was redetermined. In experiments involving peripheral administration of drugs, 100 pg HA was added to portions
of perfusion samples taken at one hour after the drugs. The percentage recovery of the external standard was used in order to assess possible interference in the assay.

Because within-group variation in basal histamine release was considerable (two-fold range), data were normalized to the percentage of mean basal values for each animal. Each drug treatment group contained four or five animals, and results are reported as the mean ± S.E.M. The results of some experiments were analysed by two-way analysis of variance (ANOVA) with time as the repeated measure.

Initial cerebral microdialysis studies were carried out to determine the effect of tetrodotoxin on extracellular histamine. Animals with implants in striatum (n = 4) or BNST (n = 5) were dialyzed as above. After the collection of four basal samples, tetrodotoxin (TTX, Sigma, 300 nM) was introduced into the perfusion solution. Thirty minute transitional samples were discarded prior to the collection of four 20 minute samples. On the day after the TTX experiments, the same animals were used in experiments to determine the calcium dependence of extracellular histamine. After the collection of four basal samples with normal perfusion solution (Ca\(^{2+}\) = 1.3 mM), animals were perfused with an otherwise identical calcium-free solution. A thirty minute transitional sample was discarded prior to collection of four further samples taken for analysis.

To study the turnover of extracellular histamine in striatum, animals were treated with the HDC suicide substrate α-fluoromethylhistidine (α-FMH, 100 mg/kg, i.p.), which irreversibly inhibits the enzyme. Samples were collected at 20 minute intervals for a period of six hours after synthesis blockade, and additional samples were collected 24 hours later.

In order to determine the effect of amino acid precursor loading on striatal histamine release, L-histidine was administered at a dose of 500 mg/kg as the free amino acid (Sigma, i.p., 200 mg/ml in 2.5% lactic acid). Samples were collected for four hours after histidine administration.

To study the effect of adrenergic drugs on extracellular histamine levels in the BNST, five 20 minute basal samples were collected, followed by the administration of the α\(_2\) agonist
clonidine (0.3 mg/kg i.p., Sigma). After collection of five further samples, the \( \alpha_2 \) antagonist yohimbine was administered (10 mg/kg i.p., Sigma) and five final samples were collected and frozen for subsequent analysis.

**C) Results**

The results of dialysis experiments in which striatum and BNST were perfused with a solution containing TTX at a concentration of 300 nM are illustrated in Figure 13. TTX, which blocks voltage-dependent sodium channels, was without effect on the extracellular histamine concentration in either striatum or BNST. Recovery of the added histamine in TTX-containing samples was quantitative. The mean basal histamine content of the dialysates were 3.8 ± 0.7 (n=14) pg/minute in the striatum and 6 ± 0.9 pg/minute (n=8) in the BNST.

The omission of Ca\(^{2+}\) from the perfusion solution (Figure 14) produced a 20% decrease in extracellular histamine levels in BNST (\( p = 0.004 \)) and also in striatum (\( p = 0.013 \)). Omission of calcium did not alter the sensitivity of the histamine assay.

After injection of \( \alpha \)-FMH there was a rapid, long-lasting disappearance of histamine outflow in striatum (Figure 15), which resolved into a bi-exponential function. The fast component, which had a half-life of 32 ± 4 minutes, was calculated from the residuals of the partial decomposition after subtraction of the slow component, which had a half-life 7.7 ± 2.2 hours. Extrapolation of the two regression lines to \( t_0 \) indicated that the rapidly turning-over pool was approximately three times larger than the slow pool. Thus, after two hours, histamine declined to 25% of basal values. At 24 hours after synthesis blockade, histamine levels had returned to 67% of basal values. The recovery of the added histamine in a dialysate taken at one hour after drug administration was 104 ± 11%, indicating a lack of interference from \( \alpha \)-FMH in the assay.

After peripheral histidine loading, histamine outflow in striatum increased to 180% of basal levels (Figure 16) over a period of one hour. Thereafter, values declined nearly to basal
Figure 13. The effect of TTX on the in vivo efflux of histamine.

After the collection of four 20 minute basal samples, the perfusion solution was modified by the addition of 300 nM TTX. The thirty minute transitional sample was discarded and four subsequent samples were collected for analysis. Data were normalized to the percentage of the mean basal value and are here reported as the mean of four separate determinations.
Figure 14. The effect of the omission of calcium from the perfusion solution on the in vivo efflux of histamine.

After collection of four 20 minute basal samples, calcium was omitted from the perfusion solution. A thirty minute transitional sample was discarded and four subsequent samples were collected for analysis. Data are normalized to the mean basal value and reported as the mean of five separate determinations.
Figure 15. The effect of $\alpha$-fluoromethylhistidine ($\alpha$-FMH) on extracellular histamine in the striatum.

After collection of three 20 minute basal samples, animals were administered $\alpha$-FMH (100 mg/kg i.p.). Values were normalized to mean basal histamine values and plotted as the mean of the natural logarithm of four separate determinations. Open squares are the residuals of the partial decomposition of the bi-exponential function with half-lives of 32 minutes and 7 hours.

The normalized histamine levels were fit to an equation of the form:

$$[\% \text{ basal HA}] = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t},$$

where:

- $A = 109, \lambda_1 = 1.33 \text{ hr}^{-1}$
- $B = 39, \lambda_2 = 0.104 \text{ hr}^{-1}$
Figure 16. Striatal extracellular histamine efflux after peripheral histidine loading.

After collection of three 20 minute basal samples, animals were administered histidine (500 mg/kg, i.p.). Data were normalized to the mean basal value and each point represents the mean of four separate determinations.
Figure 17. Histamine efflux in the BNST after administration of $\alpha_2$-adrenergic drugs.

After collection of five 20 minute basal values, animals were administered clonidine (0.3 mg/kg, i.p.), followed 100 minutes later by yohimbine (10 mg/kg, i.p.). Data were normalized to the mean basal values and each point represents the mean of four separate determinations.
values at four hours after injection. Recovery of the added histamine was 92 ± 2%, indicating
the possibility of minor interference with the assay in the presence of histidine.

In the BNST, administration of clonidine, (Figure 17), produced a 10-15% decrease in
the extracellular histamine content which just failed to reach significance (p = 0.058).
Subsequent administration of yohimbine resulted in an increased histamine signal, elevated to
155% of the mean basal levels (p = 0.047) or 180% with respect to the mean clonidine levels.
After administration of clonidine, all animals exhibited a marked behavioral sedation which
was reversed by yohimbine. Spike recovery was 97 ± 6% in the clonidine samples and 110 ±
15% in the yohimbine samples.

(D) Discussion

The present data demonstrate the presence of histamine in dialysates from the rat
striatum and BNST. In some respects, this extracellular histamine signal appears to be related
to neurotransmitter pools. However, inclusion of TTX in the perfusion solution was without
effect on histamine levels, suggesting that the extracellular histamine signal sampled by
microdialysis may be largely independant of the local action potentials. Under similar
conditions, TTX was reported to produce a rapid and complete reduction in the signal for
extracellular dopamine (Westerink et al., 1987, Drew et al., 1989), acetylcholine (Damsma et
al., 1987) and excitatory amino acids (Westerink et al., 1987). However, insensitivity of
extracellular levels of a neurotransmitter to TTX is not without precedent; the level of
extracellular GABA in striatum was unaffected by TTX (Drew et al., 1989).

Tuberomammillary neurons were spontaneously active in urethane anesthetized rats,
firing at a mean rate of 2-3 Hz (Reiner and McGeer, 1987). In rat hypothalamic slices,
histamine neurons displayed a similar rate of activity, which was blocked by the local
application of TTX (Haas and Reiner, 1988). It is therefore surprising that extracellular
histamine levels are TTX insensitive in the present study. It may be that histamine release in
striatum and BNST is largely regulated by presynaptic mechanisms such that neurotransmitter release is functionally uncoupled from the action potential. Another possible explanation for the lack of TTX sensitivity would involve the metabolism of histamine. If uptake and metabolism were in some way linked to depolarization, then local application of TTX could disable histamine clearance as well as release, producing the appearance of unaltered histamine release.

In BNST and striatum, there was a slight, but significant, decrease in histamine outflow after omission of calcium from the perfusion solution, indicating a partial calcium dependence for extracellular histamine. The potassium-evoked release of histamine from hypothalamic slices was blocked by ω-conotoxin, an antagonist of the N- and L-type voltage-sensitive calcium channels, but not by dihydropyridines which are selective for the L-type channel (Takemura et al., 1989). In striatal microdialysis experiments, omission of calcium caused the disappearance of the signals for dopamine (Imperato and di Chiara, 1984, Drew et al., 1989) and acetylcholine (Damsma et al., 1987). However, extracellular GABA in the striatum was elevated to 140% of basal levels by the omission of calcium (Drew et al., 1989) while EGTA, a calcium chelator, increased GABA outflow from synaptosomes in a manner sensitive to calcium channel blockers (Arias et al., 1984). Therefore, outflow of some neurotransmitters in the presence of reduced extracellular calcium may involve other factors, such as intracellular calcium stores.

The results of dialysis experiments after α-FMH indicate the presence of at least two pools of extracellular histamine, which is roughly in agreement with ex vivo studies conducted employing this drug. We observed that the major component of striatal histamine outflow is in a pool with a 32 minute half-life. Data reported for the disappearance of whole brain histamine in mast cell-deficient mice after administration of α-FMH (Maeyama et al., 1983) can also be analyzed to yield a bi-exponential function with half-lives of about 35 minutes and four hours.
The above data for the rapidly turning over pool of striatal histamine are in the range of half-lives reported for dopamine turnover in striatum, 12-36 minutes (Lane et al., 1982, Miyauchi et al., 1988). The half-life of histamine in striatum has previously been calculated on the basis of temporal changes in tissue content of histamine after synthesis blockade (8 minutes, Oishi et al., 1984) and by the accumulation of the metabolite r-MeHA after pargyline (11 minutes, Hough et al., 1984). However, neglecting the presence of the slow pool could lead to error in the estimation of the turnover rate for the fast pool.

The concentration of histidine in rat brain is in the order of 100 μM (Taylor and Snyder, 1972). Although the substrate affinity of HDC in vivo is not known with precision, the enzyme is evidently not saturated, as a peripheral load of histidine (500 mg/kg) increased brain histamine content by 80% (Schwartz et al., 1972). The magnitude and time course of this effect was similar to the present results for extracellular histamine in striatum. This provides further evidence for a functional relationship between total brain histamine content and histamine outflow as determined by cerebral microdialysis. The increased striatal histamine outflow observed after histidine loading was not seen in a previous study (Russell et al., 1990), a discrepancy which may have been due to the use of U-shaped cannula in an acute anesthetized preparation, rather than at least 24 hours after implantation of transtriatal probes. Westerink and de Vries (1988) have pointed out the difficulties in interpreting results from this type of acute experiment. In addition, Russell et al. (1990) employed a calcium-free perfusion solution which would, on the basis of the present results, be expected to alter the basal outflow of histamine.

A recent report on the regulation of [3H]-histamine release from cortical slices suggested that presynaptic adrenergic receptors were involved (Gulat-Marnay et al., 1989). The basal release was reported to be 40% decreased by prior administration of clonidine at 0.3 mg/kg. Yohimbine, a relatively specific α2 antagonist, was without effect alone, but blocked the clonidine-induced decrease in histamine release, suggesting that adrenergic tonus may contribute to the regulation of histamine release in the cortex. The present microdialysis data
are in agreement with the ex vivo results in that the $\alpha_2$ agonist tended to decrease extracellular histamine levels, an effect which was reversed by the antagonist. The elevation of extracellular histamine above basal levels by yohimbine administration suggests that histamine release in the BNST may be subject to tonic adrenergic inhibition in vivo. It follows that some of the physiological effects of $\alpha_2$-adrenergic drugs may be mediated via central histaminergic neurons.
VI: Histamine Receptors

(A) Introduction

As discussed in the general introduction, histamine may act through three receptor types in the nervous system. The distribution and functions of the classical $H_1$ and $H_2$ receptors have been better characterized than has the $H_3$ site, originally described as a histamine autoreceptor. In a review of the literature on the binding of histamine to cerebral membranes, it became apparent that the observations were inconsistent with the pharmacological specificities of either the $H_1$ or $H_2$ receptor (Palacios et al., 1978, Kandel, et al., 1980). In the present study the binding properties of the prototypic $H_3$ agonist $[^3H]N\alpha$-methylhistamine ($[^3H]$-N-MeHA, Arrang et al., 1983), and of $[^3H]$-histamine ($[^3H]$-HA) itself were characterized in rat forebrain sections. The two ligands were found to bind with high affinity to an identical site: the high affinity histamine binding site. Lesion studies suggest that the majority of this $H_3$ binding is associated with postsynaptic elements rather than with presynaptic histaminergic terminals.

(B) Material and Methods

Adult male Wistar rats (250-300 g) were killed by cervical dislocation and the brain quickly removed, blocked and frozen. For binding studies, 25 $\mu$m thick cryostat sections, each weighing 2.0 $\pm$ 0.1 mg, were prepared in the coronal plane. Sections were taken at the level of the greatest dimension of the striatum, mounted on chrom-alum coated glass slides and stored at -70 °C for no more than one week. Sections were thawed and then incubated for 45 minutes at room temperature in 150 mM sodium phosphate buffer (pH 7.5) containing 100 $\mu$M dithiothreitol (Sigma), 2 mM MgCl$_2$ and varying concentrations of the radioligands. $[^3H]$-HA (32 Ci/mmmole, New England Nuclear) was included at 0.4-80 nM and $[^3H]$-N-MeHA (80
Ci/mmole, New England Nuclear) was included at 0.15-25 nM. Nonspecific binding was determined by the addition of 5 mM of the H3 agonist R-(a)-methylhistamine oxalate (a-MeHA, Research Biochemicals). Sections were washed three times in 4 °C buffer (20 sec), dried under an air line, and scraped from the slides with a razor blade. The radioactivity in each section was determined by liquid scintillation counting (Packard Tri-carb 4530) after the addition of 5 ml aqueous cocktail (ACS, Amersham). The \( K_d \) and \( B_{max} \) were determined by Scatchard analysis. In some experiments, \( B_{max} \) was estimated from specific binding at radioligand concentrations ten times the respective \( K_d \)s.

To examine possible regulation of binding by guanylyl nucleotides, rat forebrain sections from three rats were incubated in the presence of 100 \( \mu \)M 5'-guanylyl-imidodiphosphate (GppNHp) in addition to \([3H]\)-N-MeHA at concentrations from 1-500 nM. At ligand concentrations between 50 and 500 nM, specific activity was reduced by the addition of unlabelled N-MeHA (Calbiochem). Non-specific binding was determined by the addition of 10 \( \mu \)M \( \alpha \)-MeHA in these experiments.

In displacement studies, radioligands were included at their respective \( K_d \)s as determined above: 8 nM in the case of \([3H]\)-HA and 2 nM in the case of \([3H]\)-N-MeHA. Displacement curves were produced by the addition of competitors at various final concentrations ranging from \( 10^{-3} \) to \( 10^{-11} \) M. The competitors used were histamine hydrochloride, mepyramine, cimetidine, and sulpiride (all from Sigma), t-MeHA (Calbiochem), impromidine (Smith, Kline, and French), harmaline hydrochloride (Aldrich), trazadone (Bristol), desmethylimipramine (Merle Dow), haloperidol (McNeil Labs), thioperamine (gift of Dr. J.-C. Schwartz) and phencyclidine hydrochloride (gift of Dr. C. Blaha).

In some animals neurochemical lesions were produced with either quinolinic acid (QA) or 6-hydroxydopamine (6-OHDA) in order to determine the anatomical localization of histamine binding. Four male Wistar rats (250-300 g) were placed in a stereotaxic apparatus while under pentobarbital anesthesia (60 mg/kg i.p.). Using the coordinates of Paxinos and Watson, a Hamilton syringe was placed in either the sensorimotor cerebral cortex (n = 2, AP: -
0.26, DV: -2.5-0.5, ML: -3.4) or the dorsal striatum (n = 2, AP: -0.26, DV: -5.5, ML: +3.4) and
1 µl of 300 mM QA (Sigma, pH 8) was infused. One rat had both cortical and striatal lesions.
QA-lesioned animals were sacrificed four days after surgery at which time neuronal
destruction is complete in the zone of the lesion while axon terminals from outside the lesion
remain largely intact (Schwarcz et al., 1983, Shaw et al., 1989). Four other animals received 1
µl of 18 mM 6-OHDA (Sigma) in ascorbic acid solution directed towards the left medial
forebrain bundle (MFB, AP: +4.5, ML: +1.5, DV: +1.5) and were sacrificed seven days after
the 6-OHDA lesions. Portions of striatum from the 6-OHDA lesioned animals were taken for
determination of catecholamine content by reversed phase HPLC with electrochemical
detection as described previously (Cumming et al., 1988).

In the lesion studies, 25 µm cryostat sections were prepared at the level of striatum and
mesencephalon. Slides containing four sections each were incubated in [3H]-N-MeHA at
concentrations of 0.5, 1, 2, 5, and 12 nM and washed as described above. The saturation
binding curves in forebrain hemisections from some 6-OHDA-lesioned animals were
determined directly by liquid scintillation counting. Other slides were exposed to tritium-
sensitive film (Amersham Hyperfilm) for a 6 week period after which time the films were
conventionally developed and fixed. Regional optical densities were quantified by
autoradiographic image analysis (Imaging Research Inc., St. Catharines, Ontario) employing
calibrated standards (Amersham).

(B) Results

A representative Scatchard analysis of [3H]-HA binding in rat forebrain (Figure 18)
yielded a K_d of 8 nM and B_{max} of 22 fmoles/section, while Scatchard analysis of [3H]-N-
MeHA binding (Figure 19) yielded a K_d of 2 nM and a B_{max} of 26 fmoles/section. The
mean of four estimations of B_{max} was 19.5 ± 2.2 fmole/2 mg section for [3H]-HA and 24.9 ±
Figure 18. Scatchard analysis of $[^3\text{H}]-\text{HA}$ binding in rat forebrain sections.

Each point represents the mean of four determinations which differed by less than 10%. Inset: the corresponding saturation binding curves.
Figure 19. Scatchard analysis of $[^{3}\text{H}]-\text{N-MeHA}$ binding in rat forebrain sections.

Each point represents the mean of four determinations which differed by less than 10%. Inset: the corresponding saturation binding curves.
Ligand Bound (fmole/section)

$B/F \times 10^6$

$[^3H]-N$-methylhistamine

(DPM/section) $\times 10^{-3}$

Ligand Concentration (nM)

Ligand Bound (fmole/section)
Figure 20. Hill plots of the binding of $[^3\text{H}]$-N-MeHA and $[^3\text{H}]$-HA to cryostat sections of rat forebrain.

Binding data were taken from the previous two figures.
\[ \log_{10} \left[ \frac{B}{(B_{\text{max}} - B)} \right] \]

\[ [^{3}\text{H}]-\text{N-methylhistamine} \]

slope = 0.87

\[ [^{3}\text{H}]-\text{histamine} \]

slope = 0.91

log\(_{10}\) [Ligand]
3.3 fmole/2 mg section for [3H]-N-MeHA. Hill coefficients for the two ligands did not differ significantly from unity (Figure 20), indicating a lack of cooperativity in binding.

Addition of 100 μM GppNHp greatly interfered with specific [3H]-N-MeHA binding: 94% of the high affinity binding disappeared with the residual component having an apparent 3 nM affinity (Figure 21). At ligand concentrations above 25 nM, a new binding site appeared with K_d of approximately 200 nM and B_max of approximately 50 fmole/section. Unfavorable signal-to-noise ratio at high ligand concentrations made it difficult to determine these quantities with precision. Sections incubated in parallel with 20 nM [3H]-N-MeHA bound 30 fmole/2 mg section in the absence of GppNHp, which is within the normal range of B_max.

In the displacement studies, nonspecific binding in forebrain sections was 12% of the total at 2 nM [3H]-N-MeHA and 25% of the total at 8 nM [3H]-HA. IC_{50}s for the displacement of [3H]-N-MeHA by some histamine ligands (Figure 22-A) and by various other psychoactive drugs (Figure 23) were estimated from the interpolation of the curves at half-maximal specific binding after subtraction of the nonspecific binding. The displacement of [3H]-HA by some competitors is illustrated in Figure 22-B.

Autoradiographic images of [3H]-N-MeHA binding in rat brain indicate that the highest binding was in the nucleus accumbens, striatum and substantia nigra pars compacta, i.e. the basal ganglia (see Figure 24). Maximal binding seemed to respect a dorsal/ventral gradient such that levels were two-fold higher in the nucleus accumbens than on the dorsal striatum (Figure 25). Dense binding was also noted in the globus pallidus, amygdala and pyriform cortex. In the diencephalon, moderate binding was present in the anterior hypothalamus and in the midline thalamic nuclei (Figure 24-C).

In the cerebral cortex, there was considerable heterogeneity in the maximal binding. Maximal binding in the anterior cingulate cortex was 30% higher than in the adjacent sensorimotor cortex (Table VI, see also Figure 24-A), but binding was very low in the retrosplenial cingulate cortex (Figure 24-D). Binding in the insular cortex appeared to be
Figure 21. The Effect of Guanylyl Nucleotide on $[^3\text{H}]$-N-MeHA Binding in Forebrain Sections.

Scatchard analysis of $[^3\text{H}]$-N-MeHA binding in rat forebrain sections in the presence of 100 μM 5'-guanylyl-imidodiphosphate (GppNHp). Each point is the mean of 4–8 determinations. A small fraction (4%) of total binding remained in a high affinity state (3 nM). At high ligand concentrations, a low affinity state appeared, the parameters of which could not be determined with precision because of the poor signal-to-noise ratio.
$[^{3}\text{H}]$–N–methylhistamine Binding in Presence of 100 μM GppNHP
Figure 22. The displacement of $H_3$ Ligands by Various Histaminergic Drugs.

The displacement of (A) $[^3H]$-N-MeHA, and (B) $[^3H]$-HA from rat forebrain sections by various histaminergic drugs. Each point is the mean of four separate determinations. $IC_{50}$, determined by interpolation at half-specific binding are: (Part A), (1) $\alpha$-MeHA, $IC_{50} = 0.4$ nM, (2) thioperamide, $IC_{50} = 3$ nM, (3) burimamide, $IC_{50} = 20$ nM, (4) histamine, $IC_{50} = 45$ nM, (5) impromidine, $IC_{50} = 79$ nM, (6) mepyramine, $IC_{50} = 79$ $\mu$M, (7) cimetidine, $IC_{50} = 100$ $\mu$M, and (8) $\tau$-methylhistamine, $IC_{50} = 100$ $\mu$M.

Drugs employed in the displacement of $[^3H]$-HA (Part B) are thioperamide, $IC_{50} = 2.4$ nM, histamine, $IC_{50} = 18$ nM, and mepyramine, $IC_{50} = 41$ $\mu$M.
Figure 23. The displacement of \[^3\text{H}\]-N-MeHA from rat forebrain sections by various psychoactive drugs.

Each point is the mean of four determinations which differed by less than 10%. IC\(_{50}\), determined by interpolation at half-specific binding, were: haloperidol (25 \(\mu\text{M}\)), sulpiride (45 \(\mu\text{M}\)), trazadone (45 \(\mu\text{M}\)), phencyclidine (70 \(\mu\text{M}\)), desmethyylimipramine (DMI, 100 \(\mu\text{M}\)) and harmaline (\(>100 \mu\text{M}\)).

Sections were incubated in $[^3]H$-N-MeHA (4 nM) washed and placed on film for six weeks.

(A) The normal striatum. The upper arrow indicates the anterior cingulate cortex and the lower arrow indicates the barrel field of somatosensory cortex. (B) Striatum at the same level as above after 6-OHDA lesion to the left medial forebrain bundle.

(C) Normal diencephalon. (D) The normal mesencephalon. The top arrow indicates the retrosplenial cortex and the bottom arrow indicates the boundary of primary and secondary visual cortex.

(E) Striatum after quinolinic acid lesion (F) Nissl staining of the above section.

(G) The mesencephalon after unilateral QA lesion to the right striatum. (H) Typical nonspecific binding in mesencephalon sections.
Figure 25. Binding of $[^3\text{H}]-\text{N-MeHA}$ in some regions of the basal ganglia

The binding of the $\text{H}_3$ ligand $[^3\text{H}]-\text{N-MeHA}$ is heterogeneous within the basal ganglia and related structures. $B_{\text{max}}$ is nearly two-fold higher in the nucleus accumbens than in the dorsal striatum. The binding in the substantia nigra pars compacta is similar to that in the striatum. In this animal, there was evidence of some heterogeneity in affinity state. Each point is the mean of three determinations.
The graph shows a plot of B/F x 10^6 on the y-axis against fmole/mg on the x-axis. The data points are differentiated by different symbols:

- Solid squares represent substantia nigra.
- Open squares represent dorsal striatum.
- Solid circles represent nucleus accumbens.

The graph has three lines, each representing a different data set, indicating a linear decrease in B/F with increasing fmole/mg.
almost equal to that in the dorsal striatum. In the parietal cortex, binding seemed to be
greater in the barrel fields than in surrounding cortex (Figure 24-A). In some cortical areas,
binding had a laminar distribution, with relatively higher binding in layers 1, 3 and 5.
However, there was little specific binding in layer 3 of the primary visual cortex (Figure 24-
D, arrow). There was relatively little binding in the dorsal hippocampus and moderate binding
in the dentate gyrus of the ventral hippocampus (Figure 24-C). Non-specific [\( ^3 \text{H} \)]-N-MeHA
binding was very low in the mesencephalon (Figure 24-H) and all other areas examined.

In the autoradiographic study, the unilateral 6-OHDA-lesion was without apparent effect
on the binding in either forebrain (Figure 24-B) or mesencephalon. Scatchard analysis of
autoradiographic [\( ^3 \text{H} \)]-N-MeHA binding in the dorsal striatum after a 6-OHDA lesion to the
left MFB does not suggest any effect of this treatment (Figure 26-B). HPLC analysis
indicated a 95% depletion of striatal dopamine in the 6-OHDA lesioned striata. In three
separate determinations of total specific binding to forebrain sections, the dopamine depletions
were without significant effect on binding parameters (Table V).

In the autoradiographic studies, striatal QA-lesions produced great reductions in binding
in striatum (Figure 24-E) and in the substantia nigra pars reticulata ipsilateral to the QA-
lesion (Figure 24-G). A representative Scatchard plot of autoradiographic [\( ^3 \text{H} \)]-N-MeHA
binding in dorsal striatum after a unilateral striatal QA-lesion is indicative of a major
reduction in maximal binding with no apparent change in affinity (Figure 26-B). The
treatment produced a 70% decrease in striatal B\(_{\text{max}}\) without significantly altering the apparent
affinity (Table V). Nissl-staining of the QA-lesioned forebrain section (Figure 24-F) showed
the tissue to be mostly intact, but devoid of neurons in the region of the lesion.

The effect of the QA-lesion on binding of [\( ^3 \text{H} \)]-N-MeHA in cerebral cortex is
illustrated in Figure 27. The unilateral QA-lesion in sensorimotor cortex produced a 70%
decrease in maximal binding without altering the affinity (Table V). The autoradiographic
binding in cingulate cortex ipsilateral to the lesion was not altered with respect to the
contralateral side. The autoradiographic appearance of \(^{3}\text{H}\)-N-MeHA binding in QA-lesioned cerebral cortex is illustrated in Figure 24-E.
Figure 26. The Effects of Striatal Lesions on the Binding of $[^3\text{H}]-\text{N-MeHA}$.

Scatchard analysis of $[^3\text{H}]-\text{N-MeHA}$ binding in rat striatum after (A) unilateral 6-OHDA lesions to the medial forebrain bundle and (B) unilateral QA-lesion in striatum. Each point is the mean of four determinations within an individual animal.
Figure 27. The Effects of Cortical QA-lesion on the Binding of $[^3H]$-N-MeHA.

Scatchard analysis of the autoradiographic $[^3H]$-N-MeHA binding in, from left to right, QA-lesioned sensorimotor cortex, sensorimotor cortex contralateral to the lesion, cingulate cortex adjacent and ipsilateral to the lesion, and cingulate cortex contralateral to the lesion.
Table V. The effect of neurochemical lesions on the binding of $[^3\text{H}]$-N-MeHA to rat brain.

<table>
<thead>
<tr>
<th></th>
<th>$B_{\text{max}}$ (a)</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Striatum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA-lesion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>contralateral</td>
<td>14.2 ± 2.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>ipsilateral</td>
<td>4.4 ± 1.6</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>6-OHDA-lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipsilateral</td>
<td>14 ± 0.8(b)</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>contralateral</td>
<td>13 ± 0.3(b)</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td><strong>Cerebral Cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA-lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>contralateral sensorimotor</td>
<td>6.7 ± 0.8*</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>ipsilateral sensorimotor</td>
<td>1.6 ± 0.5*</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>contralateral cingulate</td>
<td>9.7 ± 1.5</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>ipsilateral cingulate</td>
<td>9.4 ± 0.7</td>
<td>1.8 ± 0.4</td>
</tr>
</tbody>
</table>

(a) $B_{\text{max}}$, in units of fmole/mg, was determined by regional Scatchard analysis of autoradiographs, except in (b) where the binding was determined in whole forebrain sections weighing 2 mg each.

(c) $K_d$ is in units of nM. Note that the striatal $B_{\text{max}}$ and $K_d$ are similar when determined by regional autoradiography or from the binding in whole forebrain sections. Each reported quantity is the mean of three separate determinations ± SEM.

*p < 0.05, Student's two-tailed t-test
(D) Discussion

\[ ^3H \]-HA binds to a single high affinity site in forebrain with an affinity of 8 nM, in agreement with previous reports (Palacios et al., 1978, Barbin et al., 1980). The binding of \[ ^3H \]-N-MeHA also indicates a single high-affinity site, as previously reported for another H₃ agonist, \[ ^3H \]-α-MeHA (West et al., 1989). The Hill coefficients indicate lack of cooperativity in the binding of both ligands used in the present study. The addition of GppNHP greatly reduced the number of high affinity binding sites for \[ ^3H \]-N-MeHA as has been reported for \[ ^3H \]-HA (Barbin et al., 1980) and \[ ^3H \]-α-MeHA, (Arrang et al., 1990). Previously, GppNHP has been shown to decrease the affinity of \[ ^3H \]-HA for membranes by accelerating \( k_{off} \) (Cybulsky et al., 1981). The signal-to-noise ratio in the present study was such that the low affinity \( K_d \) and \( B_{max} \) values for \[ ^3H \]-N-MeHA binding could not be determined with precision in the presence of GppNHP. However, the present results are evidence for linkage of the majority of the \[ ^3H \]-N-MeHA binding sites to a G-protein. It is not known which second messenger systems may be coupled to the H₃ binding site, although the effects on synthesis and release are sensitive to the extracellular \( Ca^{2+} \) concentration (Arrang et al., 1985).

In the present study the most potent displacers of \[ ^3H \]-N-MeHA binding (Figure 21) were the H₃ agonist α-MeHA (IC\( _{50} \) = 0.4 nM) and the H₃ antagonist thioperamide (IC\( _{50} \) =3 nM). Impromidine, an H₂ and H₃ agonist, had an IC\( _{50} \) of 79 nM, while burimamide, an H₂ and H₃ antagonist, was a relatively more potent displacer of \[ ^3H \]-N-MeHA (IC\( _{50} \) = 20 nM). r-Methylhistamine, the ring-methylated histamine metabolite, had little ability to displace \[ ^3H \]-N-MeHA. The displacement curves indicate that \[ ^3H \]-N-MeHA behaves as a typical and potent H₃ ligand, the rank order of the IC\( _{50} \)'s for various compounds being similar to those in previous reports employing \[ ^3H \]-α-MeHA (Arrang et al., 1983, Arrang et al., 1987, West et al., 1989).

The pharmacological specificity of \[ ^3H \]-HA binding has previously been found to lack correlation with classical H₁ and H₂ sites (Barbin et al., 1980, Kandel et al., 1980). Inhibition
of $[^3H]$-HA binding to rat cortex correlated better with the subsequently characterized $H_3$ properties of some drugs than with pharmacological potency at $H_2$ sites (Steinberg et al., 1985). In the present study, $[^3H]$-HA was displaced by histamine, the selective $H_3$ antagonist thioperamide (Arrang et al., 1987) and mepyramine at IC$_{50}$ values very similar to those determined for the displacement of $[^3H]$-N-MeHA by the same competitors. In addition, the $B_{max}$ values determined for the two radioligands in forebrain sections were nearly identical. Therefore, we conclude that the high affinity histamine binding site is identical to the $H_3$ site.

The psychomimetic substance phencyclidine is able to displace $[^3H]$-N-MeHA binding with an IC$_{50}$ of 70 $\mu$M, which is similar to its reported ability to displace $[^3H]$-$\alpha$-MeHA (Arrang et al., 1988). The other psychoactive competitors in this study, including both typical and atypical antidepressants and neuroleptics, were all able to displace the $[^3H]$-N-MeHA binding with IC$_{50}$s in the range of 25-100 $\mu$M, with the exception of harmaline, a hallucinogen. As it seems unlikely that such high concentrations could be attained at normal dosages, it is improbable that important effects of these drugs could be mediated by the $H_3$ receptor. However, since these drugs were moderately potent in spite of their differing structures, it remains a possibility that some psychoactive drugs may be found to interact significantly with $H_3$ receptors.

Examination of a single autoradiogram of $[^3H]$-$\alpha$-MeHA binding suggested that high levels were present in the striatum and substantia nigra (Arrang et al., 1987). Likewise, studies of $[^3H]$-HA binding in rat brain regions indicated highest binding in striatal tissue (Palacios et al., 1978, Barbin et al., 1980). The present autoradiographic study confirms and extends these observations. In the present lesion studies, destruction of the striatal catecholaminergic innervation with 6-OHDA was without effect on striatal binding, whereas binding was greatly reduced after destruction of neurons resident in the striatum with QA. Similarly, Barbin et al., found $[^3H]$-HA binding in striatum to be 50% reduced by a local kainic acid lesion (1980). Thus, much of the striatal $H_3$ binding appears to be on intrinsic neurons, rather than on terminals of dopaminergic afferents.
The pattern of distribution of H3 binding in cortex is complex, varying from one cortical area to another and across the cortical laminae. QA-lesions, if presumed to be without great effect on presynaptic terminals, showed the majority of the [3H]-N-MeHA binding to be on intrinsic cortical neurons. A small fraction of the total H3 binding sites in the forebrain could be on afferent terminals.
VII: General Discussion

(A) Neurotoxicology

Administration of MAM on prenatal day 15 was apparently unable to prevent the development of the forebrain histamine innervation. The increases in tissue histamine levels that were detected are more reflective of decreased mass of the target structures rather than damage to histamine neurons. Similarly, Jonsson and Hallman (1982) found a two-fold increase in the rat forebrain content of serotonin, dopamine and noradrenaline after prenatal MAM treatment. These results were interpreted as evidence that MAM treatment results in a "hyperinnervation in the atrophic regions" without reduction in the total number of monoaminergic terminals. The choice of time for the MAM treatment seemed appropriate given what is known about the ontogeny of the histamine neurons, which have the peak final mitosis on embryonic day 16 (Reiner et al., 1988). Therefore, it is not clear why MAM treatment on day 15 was unable to interfere in the subsequent development of the histamine innervation. Treatment with anti-mitotic agents prior to embryonic day 15 might interfere more effectively with the subsequent development of the HA innervation.

It is interesting that MAM treatment was able to reduce HNMT levels greatly in the adult rat. The lineage of HNMT-containing cells, either neurons or glia, seems to have been largely sensitive to MAM-treatment on day 15. Alternately, expression of the catabolic enzyme in the adult may have been down-regulated in compensation for a damaged histamine innervation. Better characterization of the cellular distribution of HNMT in the adult would be pertinent to the observed MAM-senstivity.

Location of the MAO-B relevant to neurotoxicity of MPTP remains unknown, but may be in astrocytes, and/or serotonin and histamine neurons (Vincent, 1989). Although these neurons are not destroyed by MPTP, they may nonetheless contribute to the formation of MPP⁺ without themselves accumulating toxic concentrations of the metabolite. Pretreatment
with fluoxetine, a 5-HT uptake inhibitor, is reported either to protect against (Brooks et al., 1988), or to be without effect on (Melamed et al., 1985), MPTP-induced dopamine lesions. Also, prior lesions to the dorsal raphe 5-HT neurons with 5,7-dihydroxytryptamine did not attenuate the MPTP toxicity for dopamine neurons (Melamed et al., 1986). If both histamine and 5-HT neurons could be selectively destroyed, it would be possible to ascertain the contribution of MAO-B in these neurons to the production of neurotoxic MPP⁺.

Although MAO-B in histamine neurons may contribute to formation of MPP⁺, the mouse brain HA concentrations were unaffected by MPTP doses sufficient to deplete striatal dopamine greatly. The cerebral activity of HDC, the enzyme which synthesizes HA, is reported to be similar in cases of Parkinson’s disease and in post mortem specimens from neurologically normal patients (Garbarg et al., 1983). Thus, the subacute MPTP model in mice resembles Parkinson’s disease with respect to the apparent preservation of histamine in the central nervous system.

(B) HNMT Inhibition

HNMT is inhibited by a wide variety of compounds. Indeed, in an initial study of the enzyme, chlorpromazine and bromo-lysergic acid diethylamine were found to be HNMT inhibitors (Brown et al., 1959). Among the other compounds which inhibit HNMT are the biogenic amine, dopamine, and the amine metabolite, N,N-dimethyltryptamine (Sellinger et al., 1978). Other HNMT inhibitors include H₁ antagonists such as mepyramine (Taylor and Snyder, 1972, Tachibani et al., 1988), impromidine, an H₂ agonist and H₃ antagonist (Beaven and Roderick, 1980), other thiourea derivatives (Beaven and Shaff, 1979), and zolantidine, a benzthiazole H₂ antagonist (Hough et al., 1988). Among the most potent HNMT inhibitors are the dihydrofolate reductase inhibitors such as metoprine (Duch et al., 1980), antimalarial drugs such as quinacrine, which contain the aminoquinoline ring structure (Harle and Baldo, 1988), and THA, an anticholinesterase (Cumming et al., 1990).
The present *in vitro* study shows THA and metoprine to be nearly equipotent HNMT inhibitors; few compounds are more potent. Among these are quinacrine, which inhibits HNMT from guinea pig skin with a $K_i$ of 20 nM (Tachibana et al., 1988) and TMQ, an aminoquinazoline, which inhibits bovine brain HNMT with a $K_i$ of 7 nM (Duch et al., 1980). The mechanism of HNMT inhibition by THA should presumably involve structural features common to these molecules. HNMT is inhibited by a plethora of compounds with different structural features, but many of the most effective inhibitors have in common an aromatic nitrogen and a basic nitrogen separated by one or more carbons (Harle and Baldo, 1988, Duch et al., 1980, Tachibana et al., 1988). These appear to be necessary but not sufficient requirements for HNMT inhibition, since 4-AP was 400 fold less potent than THA. It may be that, for high affinity inhibition, a side-chain on the aromatic ring or the presence of an ethylamine moiety is necessary (Tachibani et al., 1988).

From the studies of HNMT inhibition, it is clear that the catalytic region of the enzyme has complex properties. A ping-pong mechanism has been proposed involving transfer of methyl from SAM to the enzyme (Thithipandha and Cohn, 1978), but because the methylated enzyme has not been isolated, direct transfer of methyl to histamine has also been suggested (Gustafsson and Forshell, 1964). Others have determined an apparent "ordered Bi-Bi" type reaction mechanism for rat brain HNMT (Orr and Quay, 1978) in which histamine and SAM bind sequentially to the enzyme. In this model, first methylhistamine and then S-adenosylhomocysteine dissociate from the enzyme after catalysis. The precise mechanism of action of the enzyme has bearing on the sites of action of enzyme inhibitors because inhibitors could potentially interfere with the binding of histamine, SAM, or both molecules.

Many inhibitors which are competitive with respect to histamine seem also to be nearly equipotent competitive inhibitors with respect to SAM (Tachibani et al., 1988). Because two molecules cannot simultaneously occupy the identical site, one cannot conclude that SAM and histamine share a common site on the enzyme, but it seems possible that HNMT inhibitors compete with the binding of both histamine and SAM by virtue of structural features common
to both substrates. One conformation of histamine (Figure 9, inset) has similarities with the adenine moiety of SAM. Harmaline also has some structural similarities with adenine (Figure 9, inset), but has a 1-methyl substitution. If β-carbolines inhibit HNMT by interfering with binding of the adenine moiety of SAM, then 1-amino analogs would be expected to be even more potent HNMT inhibitors.

Harmaline and various other β-carbolines are natural products with a number of physiological actions. Harmaline is a reversible MAO-A inhibitor with an IC$_{50}$ on the order of 10 nM (Nelson et al., 1979). Some β-carbolines, such as harmane and harmine, inhibit benzodiazepine binding with IC$_{50}$ values from 10-100 μM, although norharman-3-carboxylic acid ethyl ester is much more potent (Fehske et al., 1981). In addition, harmine and harmaline displace GABA binding from membranes with IC$_{50}$s on the order of 10 μM (Roberts et al., 1978). All the β-carbolines used in the present study inhibit dopamine uptake into rat striatal synaptosomes with IC$_{50}$ values in the range of 10-100 μM (Drucker et al., 1990). The present results indicate that β-carbolines have an ability to inhibit HNMT which is intermediate in the range of potencies for these previously described pharmacological properties.

Harmaline and related compounds may be extracted from plants such as Peganum harmala and members of genus Banisteriopsis. Such extracts are reported to be hallucinogenic and have been employed as such among some inhabitants of South America (Schultes, 1969). Harmaline is found to be a competitive inhibitor of HNMT with respect to both histamine and SAM at concentrations in the μM range. Drugs are seldom entirely specific in their actions, and inhibition of HNMT is a property of many alkaloids and synthetic nitrogenous compounds. Some compounds, such as β-carbolines, interact with such a wide variety of neurochemical systems that it is difficult to be certain which are responsible for particular physiological actions. In addition, many therapeutic agents and experimental substances such as THA are also HNMT inhibitors. Therefore, it is important to consider effects on histamine metabolism in the central nervous system as possible contributing factors to the side effects of medication and the actions of experimental substances. For example, the antimalarial
prophylactic drug mefloquine, a probable HNMT inhibitor, has been reported to produce neuropsychiatric side effects, including psychosis (Stürchler et al., 1990), which might possibly be related to effects on histaminergic transmission.

Metoprine, a dihydrofolate reductase inhibitor derived from diaminopyrimidine, is a very potent inhibitor of HNMT (Duch et al., 1980). Peripheral administration of 10 mg/kg metoprine has been previously shown to inhibit HNMT in the rat brain by more than 80% (Hough et al., 1986) and produce a long-lasting twofold increase in whole rat brain histamine levels (Duch et al., 1978). The rates of accumulation of histamine after metoprine administration, if assumed to be linear, may be calculated from data in Table V. This yields rates of 0.31 nmole/g-hour for cortex, 0.21 nmole/g-hour for striatum, and 0.71 nmole/g-hour for hypothalamus. These turnover rates are similar to, but somewhat lower than, those rates calculated from the accumulation of \( \tau \)-MeHA subsequent to pargyline administration (Oishi et al., 1984, Hough et al., 1984). The inhibition of MAO by pargyline, an irreversible inhibitor, may be more rapid and complete than the competitive inhibition of HNMT by metoprine, resulting in a probable underestimation of turnover as calculated from the accumulation of histamine after partial inhibition of HNMT.

Alzheimer's disease is a dementia often associated with degeneration of the cortical cholinergic innervation (Coyle et al., 1983) in addition to the classical neuropathological features such as neuritic plaques and tangles. The belief that a cholinergic deficit contributes to the clinical state in Alzheimer's disease has led to attempts at neurotransmitter replacement therapy, in analogy to the use of L-DOPA for Parkinson's disease. Recent interest has focused on inhibition of acetylcholinesterase (AChE), the enzyme which inactivates acetylcholine. THA inhibits AChE with an IC\(_{50}\) on the order of 100 nM (Kaul, 1962). Improved cognitive performance has been reported in some mildly demented Alzheimer's patients undergoing clinical trials with THA (Summers et al., 1986).

It is not certain that the inhibition of AChE is the only property of THA pertinent to its reported clinical efficacy and/or side effects. For example, THA shares with its structural
fragment 4-aminopyridine (4-AP) the ability to block certain classes of potassium channels (Rogawski, 1987), producing spike broadening, which could possibly potentiate various neurotransmitter systems. THA and related molecules are also inhibitors of monoamine oxidase (Kaul, 1962). These latter properties of THA, occurring at concentrations on the order of 100 μM, seem unlikely to be significant in clinical studies employing oral THA doses in the range of 100-200 mg per diem (Summers et al., 1986).

Oral physostigmine is apparently without benefit for Alzheimer's disease (Stern et al., 1987). If THA is a superior treatment to other AChE inhibitors, additional physiological and biochemical properties of THA could be responsible. The $K_i$ of THA for HNMT is well within the therapeutic range of plasma THA concentrations, which was on the order of 100 nM (Summers et al., 1986). The low dose of THA used in the present pharmacological study was able to inhibit HNMT substantially in the cerebral cortex and also to produce robust increases in cerebral histamine levels. Thus, potentiation of cerebral histaminergic transmission could be an important aspect of the actions of THA on the central nervous system.

The cholinergic deficit may be sine qua non for Alzheimer's disease, but degeneration of other non-thalamic cortically-projecting neuron systems, such as the noradrenergic locus coeruleus, is well documented (German et al., 1987). In Alzheimer's cases, high densities of neurofibrillary tangles have also been found in the vicinity of the cortically-projecting histamine neurons (Saper and German, 1987). Cortical histamine levels have been reported to be either reduced (Mazurkiewicz-Kwilecki and Nsonwah, 1989) or increased (Cacabelos et al., 1989) in studies of Alzheimer's cases. The reason for this disagreement is not clear at this time. However, it is possible that a histaminergic dysfunction may contribute to aspects of the clinical condition. Potentiation of cerebral histaminergic transmission by THA, especially in conjunction with AChE inhibition, may improve cognitive performance.
The presence of mast cells in brain has often been cited as a possible interference in the study of neuronal histamine. In a number of rodent species, mast cells are present in variable numbers in forebrain structures, especially in the dorsal thalamus, where they were in close association with blood vessels (Dropp, 1972). In the thalamus of individual adult rats, histamine content correlated highly with mast cell numbers, which ranged from 1,000-30,000 (Goldschmidt et al., 1985). However, in one mast cell-deficient strain of mice, total brain histamine content was unrelated to the mast cell phenotype (Orr and Pace, 1984). It seems that mast cell populations in brain may reflect idiosyncratic developmental processes subject to variability in different brain regions of an individual and within strains.

The residual histamine in brain several hours after synthesis blockade with α-FMH is often considered to be present in mast cells (Oishi et al., 1988). However, histamine in neonatal rat brain mast cells is reported to turn over with a four day half-life (Martres et al., 1975), which is 15-fold longer than the apparent half-life for the slow pool of extracellular histamine determined in the present striatal microdialysis experiments. Histamine leakage from immature mast cells (WoldeMussie et al., 1986), or from the leakage of blood and/or basophiles into the region of the dialysis probe, could be a factors in these experiments, given the possibility of acute trauma associated with probe implantation. However, the rapid decline in striatal histamine outflow after systemic administration of a histamine synthesis inhibitor is strong evidence for neurogenic origin. The factors regulating mast cell exocytosis are complex, but increased intracellular calcium is a necessary step (Douglas and Kagayama, 1977). Thus, the single criterion of calcium sensitivity is insufficient to discriminate between the possible neuronal and mast cell contributions to extracellular histamine. In the absence for any evidence to the contrary, the most simple explanation would be a two compartment model for cerebral histamine: fast and slow turnover neuronal pools, as has been suggested for striatal dopamine (Ewing et al., 1983), or a rapid neuronal pool and a slow mast cell histamine pool.
However, a temporal control would be necessary to determine if the apparent slow pool was simply related to declining basal histamine release.

Some of the pharmacological results here presented support the neurogenic hypothesis for extracellular histamine in the rat striatum and BNST. However, a number of interesting points may be raised. The first concerns the levels of histamine detected in dialysate samples. In striatum the outflow was close to 4 pg/min. Given that recovery of molecules by this type of transstriatal dialysis probe is proportional to molecular weight (Nomikos et al., 1990), the recovery of histamine in striatum may be estimated to be 25%; this extrapolates to an extracellular concentration for histamine of about 50 nM, which is comparable to that seen for dopamine in the striatum. However, the total tissue concentration of histamine in striatum is approximately 300 fold lower than that of dopamine (Cumming et al., 1989), perhaps relating to the lack of uptake mechanisms for extracellular histamine.

The dialysis membrane area was two times greater in striatum than in BNST. Thus, the outflow of 6 pg/min in BNST would correspond to 12 pg/min for a probe as large as that used in the striatum. Histamine outflow was greater in BNST than in striatum, as one would expect on the basis of the relatively sparse histamine innervation of the striatum (Watanabe et al., 1984). Russell et al. (1990) found histamine outflow to be greater in hypothalamus than in striatum, but absolute values cannot be compared because of the differing probe geometry and experimental design used in the two studies. Furthermore, the recovery of histamine by probes used in the present study has not been determined.

The histamine outflow was insensitive to the local application of TTX and only somewhat sensitive to the reduction of calcium around the probe. These results may reflect the range of penetration of treatments, such as local application of TTX or local removal of calcium, as compared to the tissue volume from which histamine is sampled. Histamine, lacking an uptake system, may be free to diffuse in from a range greater than that perturbed by the calcium and TTX treatments. The present study suggests that the extracellular space might be tonically flooded with histamine, such that levels are insensitive to the local
application of TTX. This hypothesis could be tested by the infusion of TTX into one medial forebrain bundle. The histamine efflux, simultaneously determined in the ipsilateral striatum should be responsive to the complete disruption of the action potential in ascending histamine fibres.

Extracellular levels of histamine in striatum are higher than what might be expected on the basis of the sparse density of innervation. Thus, the contribution of factors such as catabolism and glial uptake towards net histaminergic tonus requires further study. Given the importance of such factors as dialysis buffer composition and mode of probe implantation, the experimental conditions must be carefully chosen so as to resolve the uncertainty regarding the origin of extracellular histamine. Histamine may be behaving as a neurohumoral transmitter, acting at a considerable distance from the point of release in some brain regions.

A major limitation of the present cerebral microdialysis study was the problem of possible interference with the radioenzymatic assay. As discussed above, a wide variety of substances are inhibitors of HNMT. HNMT inhibitors, which might greatly increase the extracellular histamine levels, are certain to interfere with assay. Other molecules potentially capable of altering histamine release, H₃ drugs for example, are also likely to interfere in the radioenzymatic assay. It is not always possible to correct determined histamine concentrations for the effect of HNMT inhibition and it is difficult to attempt purification of the samples in order to remove inhibitors. The use of better analytical techniques, such as HPLC with post-column derivatization (Yamatodani et al., 1985), could facilitate the more complete characterization of the outflow of histamine in cerebral microdialysis. In particular, the effects on histamine release in vivo of drugs known to alter histamine turnover should be determined.
Histamine Binding

Pharmacological studies have previously indicated that the H3 receptor could inhibit the release of serotonin (Schlicker et al., 1988) and noradrenaline (Schlicker et al., 1988) in cerebral cortex and could inhibit the acetylcholine-mediated contraction of the trachea following vagal stimulation (Ichinose et al., 1989). Thus, H3 receptors in the nervous system cannot be solely autoreceptors. Indeed, Barbin et al. (1980) found a small increase in [3H]-HA binding sites in striatum after a 60% destruction of the histamine innervation. This is consistent with the largely post-synaptic localization of H3 receptors in brain observed in the present study.

The structural requirements for H3 activity and specificity have not been fully elucidated, but it should be noted that impromidine, which contains a guanidine moiety, is also an H2 agonist, while burimamide, which contains a thiourea, is a weak H2 antagonist (Timmerman, 1990). The possibility that some compounds may act through multiple histamine receptors must be a consideration in the interpretation of pharmacological studies. Other compounds, such as the H2 antagonist cimetidine and the H1 antagonist mepyramine, are less likely to interact with the H3 site. An additional factor in the pharmacology of histamine is that some histamine receptor ligands could alter histaminergic transmission indirectly by inhibiting HNMT, the catabolic enzyme.

[3H]-N-MeHA binding has recently been reported to bind to a single high-affinity site in membranes from guinea pig brain (KD = 0.4 nM), which, on the basis of competitive binding studies, was found to be an H3 site (Korte et al., 1990). Korte et al. (1990) reported [3H]-N-MeHA binding to be greater in the cortex than in striatum. This difference from the result reported here could reflect species differences or the use of membranes rather than brain sections. The rank order of displacers of [3H]-N-MeHA reported by Korte et al. (1990) was also partially reversed with respect to the present results. In guinea pig membranes, thioperamide, an H3 antagonist, was a less potent displacer than was histamine, an agonist.
Burimamide, another H₃ antagonist, was a less potent displacer of [³H]-N-MeHA than impromidine, an agonist. These differences from the present results may be related to the composition of buffers; whereas Korte et al. (1990) employed Tris-HCl, we used 150 mM sodium phosphate, suggesting that H₃ antagonist binding may be favored in the presence of sodium, as has been shown for the binding of opiate antagonists (Pert and Snyder, 1974). This would have interesting implications for the mechanism by which ligand binding is able to transfer information to H₃ receptors. Whereas the binding of opiate receptor antagonists is driven by a change in enthalpy (Hitzmann et al., 1985), dopamine D₂ antagonist binding can be driven by changes in either entropy or enthalpy (Testa et al., 1987). Van't Hoff analysis of the temperature dependence of H₃ binding would reveal the thermodynamic factors involved in ligand binding. If the entropic changes following receptor-ligand interactions are related to the organization of water molecules on the binding site, the addition of sodium, a chaotropic agent, might specifically influence the binding of H₃ drugs for which the dominant thermodynamic factor involved an increase in entropy. Such a phenomenon could be useful in the screening for potential agonists and antagonists.

The present autoradiographic results indicate that the highest levels of H₃ binding were in the basal ganglia. The QA-lesion produced a nearly complete destruction of specific H₃ binding in the striatum and in the ipsilateral substantia nigra pars reticulata. Therefore, the H₃ binding site is present in large part on striatonigral projection neurons. These neurons are known to contain GABA and the neuropeptides dynorphin and substance P (Semba et al., 1987, review). It follows that histamine and H₃ drugs could potentially regulate the release of some of these neurotransmitters and thus directly modulate outflow of the basal ganglia. A sparse histaminergic innervation of the striatum has been observed (Steinbusch et al., 1986). In an electron microscopic study, histidine decarboxylase-immunoreactive axonal varicosities were sometimes seen on spines, i.e. medium spiny striatonigral neurons (Takagi et al., 1986). One might therefore expect H₃ drugs to have behavioral effects related to actions on the basal ganglia. Indeed, intra-accumbens injections of N-MeHA and other H₃ agonists have been
found to produce thioperamide-sensitive hypoactivity in rats (Bristow and Bennet, 1988a, 1988b). Thioperamide enhances wakefulness in the cat, while H₃ agonists increase slow wave sleep (Lin et al., 1990), although effects on sleep-waking parameters need not be mediated by the basal ganglia.

The regional distribution of H₃ binding in rat brain is different from that of the other two histamine binding sites. In contrast to the H₃ distribution, the binding of [³H]-mepyramine, an H₁ ligand, is relatively low in the dorsal striatum and high in hypothalamic structures (Palacios et al., 1981). A selective photoaffinity ligand for the H₂ site (Ruat et al., 1990) labels most intensely the striatum and the superficial layer but not deeper layers of the cerebral cortex. In that the basal ganglia have few histamine-containing fibres, the pattern of histaminergic innervation of the forebrain (Steinbusch et al., 1986) does not match the distribution of H₃ binding. The functions of histamine in the brain must eventually be understood in terms of the polymorphic distribution of different binding sites.

We have estimated using cerebral microdialysis the extracellular concentration of histamine to be 50 nM in striatum. Although the influence of GTP on the affinity of histamine binding in vivo is not known, it seems possible that the affinity of histamine for the H₃ site could be in the range of this extracellular histamine concentration. Thus, the H₃ receptor may be an important site for the post-synaptic actions of histamine in the basal ganglia and other areas.

The functions of histamine in the brain must be understood in the light of post-synaptic functions. For example, the specific G-protein(s) involved in signal transduction at the H₃ receptor must be determined in order to clarify the functional roles of histamine. Systems known to be regulated by histamine, such as the neuroendocrine axis, have mostly been studied through the use of drugs more or less specific for the classical H₁ and H₂ receptors. Therefore, the contribution of H₃ receptors to the regulation of the neuroendocrine system is as yet unknown. In general, many of the previous behavioral and pharmacological studies in which histamine and other non-specific histamine ligands have been administered must be
reassessed in the context of the complex pharmacology of histamine binding in the central nervous system.
VIII REFERENCES


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