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The histamine H_3 receptor in the rat brain

Pharmacological and (patho)physiological aspects

Alexandra Alves Rodrigues

VRIJE UNIVERSITEIT

THE HISTAMINE H3 RECEPTOR IN THE RAT BRAIN

Pharmacological and (patho)physiological aspects

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The studies presented in this thesis were performed in the Department of Pharmacochemistry, Division of Medicinal Chemistry, Vrije Universiteit, Amsterdam, The Netherlands. The Department of Pharmacochemistry is associated with the Leiden/Amsterdam Center for Drug Research (LACDR).

THE HISTAMINE H3 RECEPTOR IN THE RAT BRAIN

Pharmacological and (patho)physiological aspects

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Chapter 1

1. General introduction

Body and soul - separated or unified?

Intensive research into neural networks, both in animals and artificial models, has created the beginnings of understanding of the biological brain machinery- an understanding of how *you* and everyone like *you* works. This idea may be found threatening, as if your innermost secrets were about to be made public. But, as Churchland (1995) states in his recent book *The Engine of Reason, the Seat of the Soul*, "you should rest assured, your physical brain is far too complex and mercurial for its behaviour to be predicted in any but the broadest outlines or for any but the shortest distances into the future. Faced with the extraordinary dynamic features of a functioning brain, no device constructible in this universe could ever predict your behaviour, or unravel your thoughts, with anything more than merely statistical success".

From the earliest speculations on the nature of man until the present day, one strongly has held the view that the body and the mind are separate, distinct entities. The prototype example of a dualist hypothesis body separated from soul (or mind) comes from Descartes who in 1664 in his *Treatise of Man* postulated the existence of the pineal gland as a site in the brain responsible for the interaction between the soul and the physical body, allowing the soul to exert control over the body. This separation of the soul/mind from the body freed the scientists of the time to work on the mechanical body without violating the "religious" soul.

As always happens in the old paths of epistemology, an opposite monist alternative has also been proposed. As early as 400 BC, the ancient Greek Democritus spoke in terms of psychic atoms responsible for thought and behaviour. Extreme versions of monist philosophy have denied either the existence of mind or its importance, stating that the "mind-type" aspects of brain functions are a by-product of the mechanistic brain.

Presently, in the era of compromise, moderate versions of monist theories which can be broadly termed as "identity" theories prevail. The Portuguese neurologist Antonio Damásio (1994) writes on his recent book *Descartes' Error* - "This is Descartes' error: the abyssal separation between body and mind, between the sizeable, dimensioned, mechanically operated, infinitely divisible body stuff, on one hand and the unsizable, undimensioned, un-pushpullable, nondivisable mind stuff; the suggestion that reasoning and moral judgement and the suffering that comes from physical pain or emotional upheaval might exist separately from the body."

This is a position also shared by Strange (1994) in his book *Brain Biochemistry and Brain Disorders* . This modern approach to body/mind dialectics states that *mind-type* aspects of brain function are the result of the total activity of the complex network of cells. In this way, mechanistic and *mind-type* descriptions of the brain describe the same neural activity but at different levels. It becomes obvious from the present state of the knowledge of the human brain, that there is a major gap between the understanding of the *mind-type* properties and its mechanistic aspects. In fact, it is this gap that will always promote dualist hypotheses. The role of the "identitist" (being him/her a scientist and/or a philosopher) is to attempt to bridge this gap with theories and experimental evidences that show how the total activity of brain cells can give rise to the *mind-type* aspects of the human brain. In fact, modern approaches to brain disorders have shown that treatments for these malfunctions may be based on drug and psychological therapies. These kinds of treatment may be effective for, e.g., anxiety and affective disorders, where it seems likely that both therapeutic approaches are ultimately affecting the same or closely related brain systems.

Churchland prophecies on the ultimate success in predicting human behaviours and thoughts being of merely statistical nature are further supported by Penrose (1989) who proposed that quantum mechanical (probabilistic) principles need to be applied to the functions of the overall neural network in the brain in order to understand the mental functions of the brain. For example, if a neuron receives hundreds or even thousands of synaptic inputs, it seems reasonable that the functional output states of that neuron may be achieved by more than a single input configuration. This probabilistic approach can also be applied to interpret and predict the actions of neuromodulators such as histamine. This biogenic amine is the least studied of all the known bioamines and its actions in the CNS will be the focus of this dissertation.

1.1 Anatomical aspects of the CNS

Neuron and synapse

The stereotypical image of a neuron is that of a stellate cell body (soma or perikaryon) with broad dendrites and a fine axon emerging from one pole (Raine, 1994). However, this morphology does not hold true for many neurons. The neuron is the most polymorphic cell in the body and defies formal classification on the basis of shape, location, function, fine structure, or transmitter substance. Nerve cells range from the small globular cerebellar granule cells, with a perikaryal diameter of approximately 6 to 8 µm, to the pear-shaped Purkinje cells and star-shaped anterior horn cells, both of which may reach diameters of 60 to 80 µm in humans. Perikaryal size is generally a poor index

of total cell volume, however, it is a general rule in neuroanatomy that neurites occupy a greater percentage of the cell surface area than the soma (Raine, 1994).

The extent of branching displayed by the dendrites is a useful index of their functional importance. Dendritic trees represent the expression of the receptive fields and large fields can receive inputs from multiple origins. A cell with a less developed dendritic ramification (e.g. the cerebellar granule cell) synapses with a more homogenous population of afferent sources (Raine, 1994).

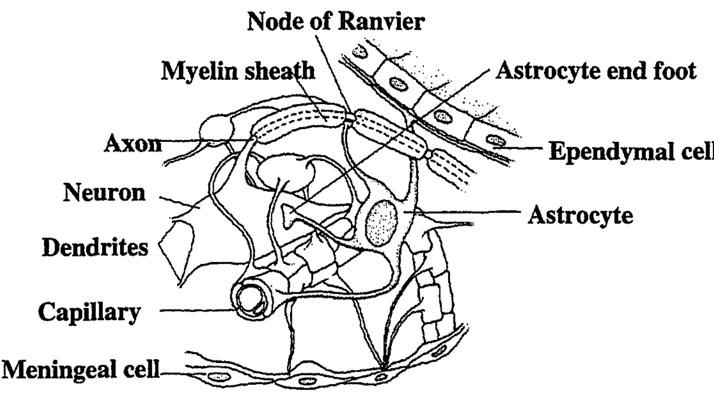


Figure 1.1 Schematic representation of the interactions between astrocytes, capillaries, and neurons in the mammalian brain.

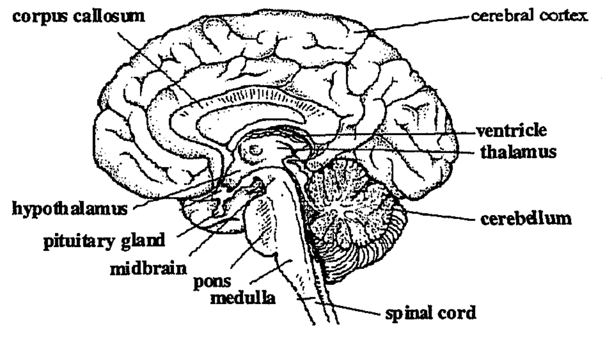
The axon emerges from a neuron as a slender thread and usually does not branch until it nears its target. In contrast to the dendrite and the soma (with very few exceptions), the axon is frequently myelinated, thus increasing its efficiency as a conducting unit. Myelin, a spirally wrapped membrane, is laid down in segments (internodes) by oligodendrocytes in the CNS and by Schwann cells in the PNS. The naked regions of axon between adjacent myelin internodes are known as *nodes of Ranvier* (Figure 1.1). When the membane at the node is excited, the local circuit generated cannot flow through the high resistance sheath and, therefore, flows out through and depolarises the membrane at the next node which might be one mm or farther away. Active excitation of the axonal membrane jumps from node to node (Morell *et al.*, 1994).

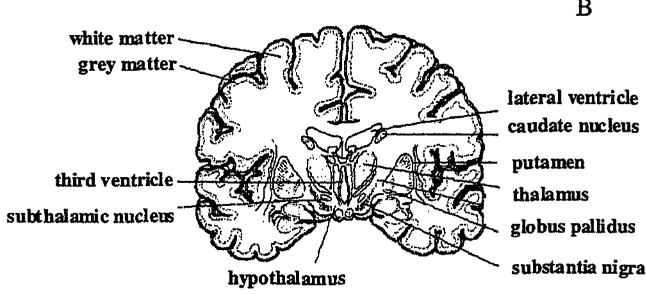
Mammalian brain anatomy

The internal structures of the human brain are shown in figures 1.2 A and 1.2 B. The mid-sagittal section (fig. 1.2 A) shows more detail of infoldings of the cerebral cortex and of the cerebellum. The entry of the brain stem into the brain and the components of

the brain stem (medulla, pons and midbrain) are visible as well as their linkage to the diencephalon (a collective name for the thalamus and hypothalamus).

One can also see in this section the corpus callosum, a set of nerve fiber tracts linking the two hemispheres, and a ventricle, a fluid-filled space within the brain.
In the coronal section, (fig. 1.2.B) the symmetry of the two hemispheres is emphasised.





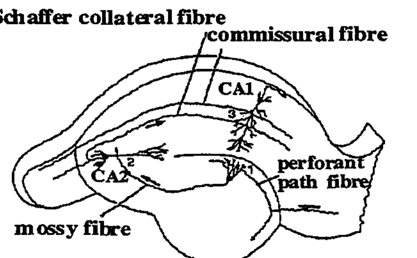


Figure 1.2 Overall structure of the human brain at the neuroanatomical level; (A) coronal section and (B) sagittal section. (C) Schematic diagram of the three major excitatory pathways in the hippocampus: 1, from the entorhinal area to dentate granular cells via the perforant path; 2, from dentate granular cells to CA3 pyramidal cells via mossy fibres; 3, from CA3 pyramidal cells to CA1 pyramidal cells via the Schaffer collateral. Commissural fibres project from the contralateral hippocampus to CA1 pyramidal cells (Nicoll *et al.*, 1988).

Further internal structure is now visible and the interrelationships of the thalamus, hypothalamus and the ventricles is clearer. Structures that appear now visible are the corpus striatum (composed of the caudate nucleus, globus pallidus, and putamen) and part of a system called the basal ganglia. An important nucleus which is not shown in these figures is the hippocampus, a subcortical bean-shaped structure present towards the caudal part of the brain, which plays a role in memory and control of behaviour (fig. 1.2.C).

Some areas of the cerebral cortex have been connected with certain aspects of the peripheral activities, e.g., motor cortex for muscle control, somatosensory cortex for sensory input, visual cortex for visual input, etc. There are also large areas of the brain which cannot be identified with specific functions and which presumably perform more complex syntheses of information; such centres are highly developed in man compared to other species. Brain functions can, therefore, be seen to be due to co-operative performance of the whole organ rather than to the isolated actions of individuals parts.

1.2 Brain neuropathology

For several brain disorders it was found that certain parts of the diseased brain are being degenerated, or in some way disrupted. Some of these brain disorders are briefly discussed in this section.

Parkinson's disease

The pathological hallmark of Parkinson's disease is now felt to be Lewy body induced degeneration of pigmented and other brainstem nuclei (*substantia nigra compacta, locus caeruleus, dorsal nuclei of the vagus, nucleus accumbens, and nucleus basalis* of Meynert). The Lewy body is an eosinophilic inclusion with a characteristic halo found in the degenerating neurons. It is made up of neurofilaments and has immunoreactivity for ubiquitin. Loss of cells by, presumably, oxidative stress from the substantia nigra in Parkinson's disease results in profound dopamine depletion in the striatum, ventrolateral projections to putamen are more affected than dorsomedial projections to head of caudate. Loss of locus caeruleus and nucleus basalis of Meynert cells leads to reduced levels of striatal and cortical noradrenaline and acetylcholine, respectively (Brooks, 1995).

Schizophrenia

While the underlying cause of Parkinson's disease is hypothesized to be 'oxidative stress' (Singer *et al.*, 1995), the underlying cause of schizophrenia is a structurally abnormal cerebral cortex (Weinberger & Lipska, 1995). The association of schizophrenia with cortical maldevelopment, has been supported by the discovery of 'hypofrontality' in some patients: blood flow in the dorsolateral prefrontal cortex is abnormally low upon

challenge with relevant cognitive tasks (Weinberger *et al.*, 1992). In addition, it has been reported that there are reduced numbers of neurons in parts of the prefrontal cortex (Benes *et al.*, 1986; 1991), and abnormalities in the distribution of nitric oxide synthase (NOS)-containing neurons in frontal white matter in postmortem tissue from schizophrenics (Akbarian, 1993a). Several irregularities have also been reported in the temporal lobes: for example, changes in the distribution of NOS-containing neurons (Akbarian, 1993b), and alterations in the cytoarchitecture and volume of the hippocampus and entorhinal cortex see (Winn, 1994) for further references). It has been proposed that changes in the location, but not the number, of these NOS-containing neurons in brains of schizophrenics reflects abnormal neuronal migration during development, which presumably would be followed by the establishment of unusual patterns of synaptic connectivity (Bloom, 1993).

Alzheimer's disease

The primary causes of Alzheimer's disease have not been elucidated, yet. The disorder was first described at the beginning of this century in a demented woman by Alois Alzheimer. Two major brain lesions were observed, which later became diagnostic for Alzheimer's disease and which are generally termed senile plaques (SPs) and neurofibrillary tangles (NFTs). Today, Alzheimer's disease is diagnosed clinically on the basis of neurological examination and neuropsychological testing indicating progressive impairment of memory and intellectual functioning. Also, brain imaging techniques show severe brain atrophy due to extensive neuronal cell loss. However, the ***diagnosis*** of Alzheimer's disease is only reliable if a histopathological examination at brain autopsy shows high numbers of SPs and NFTs particularly in the hippocampus and the cerebral cortex. Apart from the SPs and NFTs, the Alzheimer's disease brain pathology also includes congophilic angiopathy of the cerebral blood vessels (Van Broeckhoven, 1995). The SPs are extracellular deposits in the brain parenchyma made up of a core, consisting of fibrils of a protein termed β-amyloid, surrounded by dystrophic neurites (DNs). The DNs are most likely the result of a neurodegenerative response to the β-amyloid deposition since parenchymal deposits of β-amyloid in the absence of DNs are also abundantly present. The β-amyloid is also found in the walls of the small vasculature of the leptomeninges and the cerebral cortex where it is responsible for the congophilic angiopathy. The NFTs are intraneuronal inclusions composed of paired helical filaments of the microtubuli-associated protein tau, although in an abnormally phosphorylated form. *Postmortem* immunohistochemical studies have suggested that the appearance of the β-amyloid deposits in the parenchyma, the cerebral blood vessels is the earliest structural sign of Alzheimer's disease (Van Broeckhoven, 1995).

Epilepsy

Epileptic patients experience severe convulsions in certain brain areas. The underlying neuronal abnormality in epilepsy is poorly understood. It has been shown that local cortical damage results in a type of focal epilepsy. The characteristic event is the seizure, which is often associated with convulsions, but may occur in many other forms. The seizure is caused by an abnormal high frequency discharge of a group of neurons, starting locally and spreading to a varying extent to affect other parts of the brain. Seizures may be partial or generalised depending on the location and spread of the abnormal neuronal discharge. The attack mainly involves motor, sensory or behavioural phenomena. Unconsciousness occurs when the reticular formation is involved. Partial seizures are often associated with damage to the brain, whereas generalised seizures occur without obvious cause. A large variety of affections are called epilepsy. Two common forms of generalised epilepsy are the tonic-cronic fit (grand mal) and the absence seizure (petit mal). The neurochemical basis of the abnormal discharge may be associated with enhanced excitatory amino acid transmission, impaired inhibitory transmission, or abnormal electrical properties of the affected cells (Rang & Dale, 1995).

1.3 The histaminergic central neuronal system; a brief review

Histamine receptors

The early history of histamine is largely associated with allergies. The major actions of histamine were described at the beginning of this century by Sir Henry Dale and his colleagues after its isolation from ergot extracts (Dale & Laidlaw, 1910). Histamine's potent contractile effects on smooth muscles and the induced capillary dilation, which mimic some initial manifestations of the anaphylactic shock, were found by these scientists. They also detected the presence of the amine in various tissues, but it was another German scientist, Feldberg, who clearly demonstrated that histamine was released from the lung during the anaphylactic shock and that it induced a marked bronchoconstriction (Feldberg, 1927; Feldberg, 1941).

The idea that histamine exerts its various biological effects via interaction with several distinct receptor subtypes progressively arose with the design of subtype-selective antagonists. It was first observed that the "antihistamines" (now known as H_1-receptor antagonists), the first of which were designed by Bovet & Staub (1937), did not block uniformly all actions of histamine, leaving, for instance, gastric acid secretion unaffected. Based on this knowledge, as well as on the differential action of histamine and histamine agonists, Ash and Schild (1966) postulated the existence of a second receptor subtype. The existence of the H_2 receptor was definitively established with the design of burimamide, a selective H_2 (non H_1) antagonist, and with the development of several relatively selective agonists (Black *et al.*, 1972).

By the mid 70s, although H_1 and H_2 receptors were shown to mediate central histaminergic neurotransmission (Garbarg *et al.*, 1974), the histaminergic neuronal system in brain had remained largely unexplored in drug design. Only the traditional brain-penetrating drugs used for the effects mediated by H_1-receptors and available as over-the-counter sleeping pills, are drugs known to interfere with histaminergic transmission in the CNS. This fact contrasts with the emergence, during the last decade, of a detailed knowledge of the histaminergic systems revealing that it shares many biological and functional properties with other aminergic systems.

In 1983, Arrang *et al.* proposed the existence of a third receptor subtype, an autoreceptor, i.e. presynaptically localised on histaminergic nerve terminals, and modulating the release of histamine in neurons. The existence of this H_3 receptor subtype was fully established in 1987 by the same researchers when it was shown to be involved in the regulation of histamine synthesis as well (see section 2). It was initially shown in brain slices or synaptosomes after labelling the endogenous pool of histamine using tritiated histidine (Arrang *et al.*, 1987). Exogenous histamine decreases the depolarisation-induced release and formation of [^{3}H]histamine, and analysis of these responses led to the pharmacological definition of H_3 receptors. Its localisation in the brain and periphery was then revealed with the design of highly potent and selective agonists, such as R-(α)-methylhistamine, and antagonists, such as thioperamide. Autoregulation was found in various brain regions known to contain histaminergic nerve endings, suggesting that all histaminergic terminals are endowed with H_3 autoreceptors (Schwartz *et al.*, 1995). Regulation of histamine synthesis was also observed in the posterior hypothalamus, possibly indicating the existence of autoreceptors at the level of histaminergic perikarya or dendrites (Arrang *et al.*, 1992).

Metabolic synthesis of histamine

The biosynthesis and metabolism of histamine has been extensively reviewed in the last couple of years. The information presented in this section represents a summary of these reviews (Onodera *et al.*, 1994; Schwartz *et al.*, 1991). Histamine itself poorly penetrates the blood-brain-barrier (BBB) and must, therefore, be formed locally. Histamine is synthesised in a single step by the highly specific enzyme L-histidine decarboxylase (HDC, EC 4.1.1.22, figure 1.3.), and HDC inhibitors are, thus potentially useful tools to investigate the role of histamine. S(α)–fluoromethylhistidine potently inhibits in a stereoselective, time-dependent, and concentration-dependent manner cerebral HDC with an inhibitor constant [K_i] of ~10^{-5} M, whereas related decarboxylases such as dopa or glutamate decarboxylase are not significantly affected (Garbarg *et al.*, 1980). Indeed, α–FMH is a suicide substrate that remains bound to the enzyme after being decarboxylated. S(α)–fluoromethylhistidine, administered systematically in rather low dosages, rapidly, completely, and in a long-lasting manner

inactivates HDC in brain and peripheral organs (for references see Schwartz, 1991a). Restoration of the activity occurs progressively, presumably as a result of neosynthesis of HDC molecules, and in brain the process is first detected in the hypothalamus in which histamine perikarya are located (Garbarg *et al.*, 1980). Because α–FMH efficiently depletes histamine stores in cerebral neurons (Garbarg *et al.*, 1980; Maeyama *et al.*, 1982), the compound is a useful tool for investigating the amine turnover and functions therein.

It was shown by Sakai *et al.* (1992) that the H_3-receptor antagonist thioperamide significantly increased HDC activity in the brain of various strains of mice (figure 1.3.). It could not be demonstrated, however, that the H3 agonist R(α)-MeHA decreases HDC activity (Oishi *et al.*, 1989).

Histamine present in the brain is stored in neurons and mast cells. It is being released from the neurons upon depolarisation. Obviously, mast cells do not depolarise because mast cells do not posses voltage-dependent Ca^{2+} channels in their plasma membrane. The brain mast cells are, therefore, considered as slow-turnover pools and are with difficultly depleted in contrast with the neuronal storage. The function of brain mast cells is unknown and the existence of additional non-neuronal pools was postulated (Schwartz *et al.*, 1991).

[^{3}H]-Histamine release from histaminergic neurons is not only inhibited by stimulation of histamine autoreceptors but also by, e.g., α_2-adrenoreceptors, M_1-muscarinic receptors, and κ-opioid receptors (Schwartz *et al.*, 1991). Since these regulations are also observed with synaptosomes (Schwartz *et al.*, 1990), all these receptors presumably represent true presynaptic heteroreceptors. In contrast, histamine release is enhanced by stimulation of nicotinic receptors in rat hypothalamus and by μ-opioid receptors in mouse cerebral cortex (Schwartz *et al.*, 1991). Neither the opposite effects of the κ- and μ-opioid receptors, nor their physiological functioning, are well understood. Also, the physiological function of the localisation of the nicotinic receptors on the histaminergic nerve terminals remains unclear.

Inactivation of histamine

Histamine neurons seem to be almost unique among monoaminergic neurons in that they lack a high-affinity reuptake system. The only way to deactivate the histamine released is by catabolism (Schwartz *et al.*, 1991). This catabolism occurs along two alternative pathways, i.e. transmethylation into *tele*-methylhistamine (*t*-MeHA) catalysed by histamine N-methyltransferase (HMT, EC 2.1.1.8), and oxidative deamination into imidazolacetic acid (IAA), catalysed by diamine oxydase (histaminase, EC 1.4.3.6) (figure 1.3). Under normal physiological conditions, only the former pathway operates in mammalian brain; it must be noted, however, that if this route is inhibited by an HMT

inhibitor such as metoprine (Thomas & Prell, 1995), the latter pathway forming the IAA metabolite becomes important (see figure 1.3).

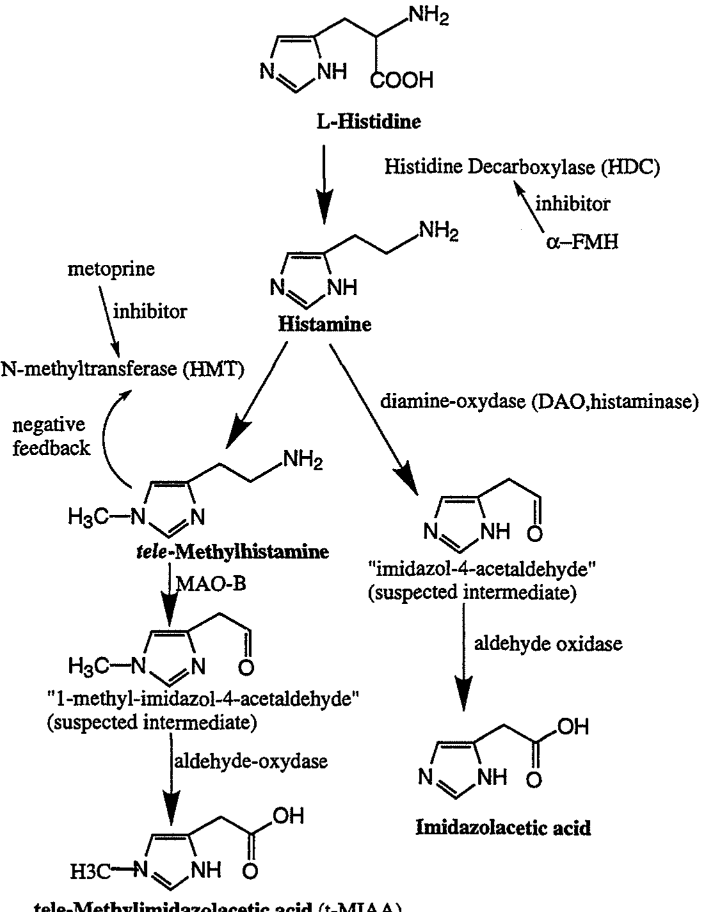


Figure 1.3 Synthesis and metabolism of histamine

The HMT activity is not only inhibited by compounds like metoprine, but also by its own reaction product *t*-MeHA, which causes an increase in histamine levels in the brain, implying a possible regulatory role (Schwartz *et al.*, 1973). Hence, another possible way to increase the level of histamine by influencing its metabolic pathway, is by inhibiting MAO-B with, e.g., pargyline which causes an increase in *t*-MeHA levels and, thus, indirectly causes increasing histamine levels as well (Schwartz *et al.*, 1991).

Cell bodies

The central histaminergic neuron system in rat brain was identified by immunohistochemical studies with antibodies against histidine decarboxylase (HDC) (Panula *et al.*, 1989; Watanabe *et al.*, 1984) or conjugated histamine as markers, and recently, by *in situ* hybridisation studies using oligonucleotides corresponding to HDC cDNA (Bayliss *et al.*, 1990; Panula *et al.*, 1990). Cell bodies of the histaminergic neuronal system are located exclusively in the tuberomammillary nucleus of the posterior hypothalamus in five cell clusters known as E1-E5.

Histaminergic neurons are relatively large (25-35 μM in diameter), and have 2-4 well developed dendrites with a few dendritic spines. Some of the cell bodies are located in the ventral surface of the brain and the dendrites seem to be in contact with the cerebrospinal fluid (CSF) (Hayashi *et al.*, 1984; Watanabe *et al.*, 1984). The membrane properties of the neurons have been studied electrophysiologically *in vitro*, using explants of rat hypothalamus (Haas & Reiner, 1988). The neurons are spontaneously active, firing at about 2 Hz; their action potentials are followed by marked after-hyperpolarizations. These morphological and electrophysiological features of the cell bodies are similar to those of other monoaminergic neurons, such as noradrenergic, serotonergic and cholinergic large neurons located elsewhere in the neuronal system (Onodera *et al.*, 1994).

The histaminergic neurons are characterised by the presence of an unusually large variety of markers for other neurotransmitter systems: glutamic acid decarboxylase, the gamma-aminobutyric acid (GABA)-synthesizing enzyme; adenosine deaminase, a cytoplasmic enzyme involved in adenosine inactivation; galanin, a peptide co-localised with all other monoamines; (Met^5)-enkephalyl-Arg^6Phe^7, a product of the proenkephalin A gene; and other neuropeptides, such as substance P, thyroliberin, or brain natuiretic peptide (Schwartz *et al.*, 1995). The functions of the putative co-transmitters remains to be established, but their presence indicates that the histaminergic system is indeed involved in whole-brain activity, and can have implications in pathophysiological conditions such as pain, anxiety, and, more generally, regulation of the overall state of neuronal and glial activity.

Afferent fibre connections

Afferent fibre connections to the histaminergic neurons from the prefrontal cortex, medial preoptic nucleus, and septum-diagonal band complex have been demonstrated by Ericson *et al.*, (1989; 1991). The details of the origin of the neuronal inputs to the tuberomammillary nucleus of the posterior hypothalamus were determined using comprehensive retrograde and anterograde tracing techniques. Their results indicated that the main afferent fibres originated from some limbic forebrain regions, such as the infralimbic cortex, septum and preoptic region. The areas associated with primary sensory or motor functions did not innervate the tuberomammillary nucleus (Onodera *et*

al., 1994). The histaminergic neurons receive synaptic contacts from varicose fibres containing neuropeptide Y, substance P and γ-aminobutyric acid (GABA). Adrenergic, noradrenergic and serotonergic afferents, are also reported to innervate the tuberomammillary nucleus of the posterior hypothalamus (Ericson *et al.*, 1989).

Distribution of efferent fibres

Efferent fibres of the histaminergic system are distributed in almost all regions of the brain from the olfactory bulb to the spinal cord in rats. The density of these fibres is highest in the hypothalamic nuclei, medial septum and diagonal band, moderate in the cerebral cortex, basal ganglia and amygdaloid complex, and low in the olfactory bulb, hippocampus, caudate-putamen, brain stem, cerebellum, spinal cord and posterior hypophysis. No fibres innervate the retina or the intermediate and anterior lobes of the hypophysis (Onodera *et al.*, 1994) (see figure 1.4).

Phylogeny of the histaminergic neuronal system

All vertebrate brains so far studied contain a significant amount of histamine possibly of neuronal origin (Miki *et al.*, 1992). Besides the rat brain, the histaminergic system has been demonstrated immunohistochemically in guinea pig (Airaksinen & Panula, 1988), tree shrew (Airaksinen *et al.*, 1989) and human brains (Panula *et al.*, 1990) and in the brains of nonmammalian vertebrates, such as the turtle (Inagaki *et al.*, 1990), frog (Airaksinen & Panula, 1990), teleost (Inagaki *et al.*, 1990), and lamprey (Brodin *et al.*, 1990). In these animals, the basic organisation of the system is the same; i.e., the cell bodies are located in the posterior part of the ventral hypothalamus and have extensive fibre projections to almost all areas of the brain. The only exception observed so far is the lamprey, which has a second group of histamine-immunoreactive neurons in the hindbrain (Brodin *et al.*, 1990).

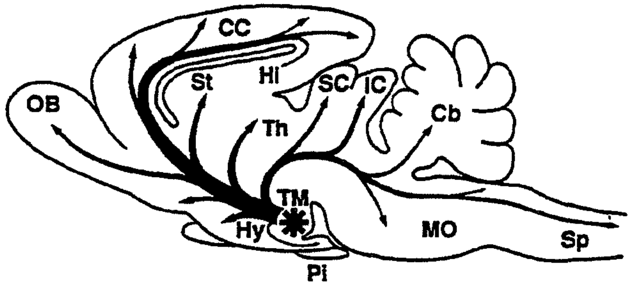


Figure 1.4. A sagittal diagram of the histaminergic system in the rat brain. Arrows indicate the fibre projection from the cell bodies (asterisk). Abbreviations: **Cb**, cerebellum; **CC**, cerebral cortex; **Hi**, hippocampus; **Hy**, hypothalamus; **IC**, inferior colliculus; **MO**, medulla oblongata; **OB**, olfactory bulb; **Pi**, pituitary; **SC**, superior colliculus; **Sp**, spinal cord; **St**, striatum; **Th**, thalamus; **TM**, tuberomammillary nucleus (Wada *et al.*, 1991).

These cells may correspond to the cell bodies that appear transiently in the lower brain stem of the rat embryo during ontogenesis (Auvinen & Panula, 1988). In general, the principal morphological features of the histaminergic neuron system appear to be well conserved among vertebrates.

Ontogeny of the histaminergic neuronal system

The ontogeny of immunoreactive histamine (irHA) (Auvinen & Panula, 1988) and immuno-reactive histidine decarboxylase (irHDC) neurons (Reiner & McGeer, 1987) was studied in rat brain. The first irHA perikarya were seen on embryonic *day 13* in the border of metencephalon and mesencephalon and on *day 15,* in the ventral mesencephalon, metencephalon, and myelencephalon. From these scattered cells a transient ascending and descending fibre system starts to develop on embryonic *day 15* but has completely disappeared on *day 20.* In contrast, in the basolateral hypothalamus, irHA (Reiner & McGeer, 1987) and irHDC cells (Reiner & McGeer, 1987) were first detected on embryonic *day 16* when they had stopped their mitotic division. The differentiation of immunoreactive neurons in the various subgroups of the caudolateral part of the tuberomammillary nucleus seems largely achieved by embryonic *day 20,* whereas the appearance of the dorsal subgroup in the medial part of the nucleus only occurs during the last prenatal days.

The development of most irHA fibres takes place during the first two postnatal weeks (Auvinen & Panula, 1988), which coincides with the developmental pattern of HDC activity taken as a selective marker of histamine neuronal pool (Martres *et al.*, 1975).
In contrast, irHA mast cells are most numerous on postnatal *day 4* (when they are mostly located in the hippocampal area), and after that, their number gradually decreases (Auvinen & Panula, 1988), a pattern that explains a decrease in histamine levels after *day 4*, as proposed by Martres *et al.* (1975).

2. The histamine H3 receptor

The histamine H_3 receptor was discovered as an autoreceptor, i.e. presynaptically localised on histaminergic nerve terminals, and controlling the synthesis and release of histamine in cerebral neurons (Arrang *et al.*, 1983; Arrang *et al.*, 1987). It was observed that, after potassium depolarisation, [^{3}H]-histamine is released from rat brain cortex slices preloaded with [^{3}H]-histidine. Exogenous histamine decreases the release and formation of [^{3}H]-histamine and the analysis of these responses led to the pharmacological definition of the H_3 receptor (Arrang *et al.*, 1983; Arrang *et al.*, 1987). Using microdialysis, push-pull canula superfusion or *ex vivo* histamine determination, several authors (Arrang *et al.*, 1987, Koss & Hey, 1992; Prast *et al.*, 1994; Taylor *et al.*, 1992)

were, subsequently, able to show *in vivo* effects of the stimulation of the H_3 receptor. The autoregulation was found in various brain regions known to contain histaminergic nerve endings, suggesting that all terminals possess H_3 autoreceptors (Koss & Hey, 1992; Prast *et al.*, 1994; Taylor *et al.*, 1992).

More recently it was found that the H_3 receptor also acts as a heteroreceptor, regulating the release of other neurotransmitters such as acetylcholine (Clapham & Kilpatrick, 1992) serotonine, noradrenaline, and dopamine both in the CNS (Schlicker *et al.*, 1988; Schlicker *et al.*, 1993; Schlicker *et al.*, 1989; Schlicker *et al.*, 1994) and in peripheral tissues such as the GI tract (Bertaccini *et al.*, 1991). Based on these findings, the H_3 receptor should be considered a potentially important regulatory centre which modulates a variety of brain (section 3) and peripheral functions.

2.1.1 Pharmacological characterisation

As the molecular structure of the H_3 receptor remains to be established, the information on this receptor has to be based on its pharmacological and physiological properties. Fortunately, the H_3 receptor has been the target for successful development of selective agonists and antagonists (Leurs *et al.*, 1995). Histamine, itself, is a quite potent H_3 agonist (e.g. Arrang *et al.*, 1983; Cumming *et al.*, 1991; Leurs *et al.*, 1995). As might be expected, most potent H_3 agonists are obtained from modifications of the histamine molecule (Table 1). Nevertheless, substituents on the imidazole ring result in a marked decrease in agonistic activity and affinity (Leurs & Timmermann, 1992), as has been found for the other histamine receptors. In contrast with the H_1 and H_2 receptors, selective and highly potent H_3 agonists have been synthesised (Leurs & Timmermann, 1992). Substitution of the side chain of histamine has resulted in the high affinity H_3 agonist (R)-α–methylhistamine (Arrang *et al.*, 1987; Arrang *et al.*, 1985), whereas replacement of the amino group with a basic isothiourea group resulted in the potent agonist imetit (Ganellin *et al.*, 1992; Garbarg *et al.*, 1992; Howson *et al.*, 1992; Leurs *et al.*, 1995; Van der Goot *et al.*, 1992). The most potent H3 agonist known so far is the compound SKF 91606 (pD_2 9.0) which showed that the sulphur atom in the isothiorea group is not essential (Howson *et al.*, 1992). The alkyl chain separating the imidazole ring and the amine group can be longer than an ethylene as proved by the potent agonist immepip (pD_2 8.0) (Vollinga *et al.*, 1994). It must be noted, however, that only a cyclic lengthened side-chain is allowed for agonistic activity. Otherwise, extension of the side-chain leads to potent histamine H_3 antagonists, such as impentamine (pA_2 8.4 in the guinea pig jejunum) (Leurs *et al.*, 1995).

The best known H_3 antagonists can be divided into four main groups. The first comprises histamine and imetit analogues, which result from an elongation of the side chain between the imidazole group and the cationic nitrogen. This group of compounds

contains two of the most potent H3 antagonist clobenpropit and impentamine (Table 1, Leurs *et al.*, 1995a). The second group of antagonists is formed by burimamide analogues (Vollinga *et al.*, 1994) in which thioperamide, the first potent H3 antagonist described (Arrang *et al.*, 1987), has been included. Although thioperamide is a burimamide analogue, structure-activity relationships have questioned the analogy between the way of interaction with the H3 receptor of thioperamide and other burimamide analogues. Therefore, thioperamide and its derivatives (e.g., GT-2016, a non-isothiourea derivative as described by Tedford *et al.*, 1995) should be included in a third group of compounds.

Table 1 Some of the most potent histamine H3 rceptor ligands.

H3-receptor Agonists	*H3 receceptor antagonists*
(R)-α-methylhistamine	Thioperamide
Imetit (VUF 8325)	Clobenpropit (VUF 9153)
Immepip (VUF 4708)	Iodoproxyfan
SKF 91606	Impentamine (VUF 4702)

Finally there is the group of compounds that show H3 antagonism, but their chemical structure does not fall into any of the above categories and their functional group is not protonated like iodoproxyfan (Ligneau *et al.*, 1994). It should be noted, however, that agonistic activity has been described for this compound (Schlicker *et al.*, 1995) making it the first H3 agonist lacking an amine function. Apparently, a lipophilic moiety separated from the imidazole ring by an alkyl spacer is sufficient for H3 antagonistic activity (Leurs

et al., 1995a). Recently clozapine, a non-imidazolic tricyclic compound, has been shown to be an H_3 antagonist (Alves Rodrigues *et al.*, 1995; Kathmann *et al.*, 1994), and preliminary studies on the structure affinity relationships have been determined for a series of clozapine analogues (Alves-Rodrigues *et al.*, 1996).

2.1.2 Biochemical aspects of the H3 receptor

Second messenger

Due to the relatively low abundance of histamine H_3 receptors in various tissues and because highly sensitive radioligands have only recently become available, the biochemical investigations of the H_3 receptor are limited. Using [^{3}H]-histamine as radioligand, the solubilisation of the bovine H_3 receptor from brain tissue was reported (Zweig *et al.*, 1992). The apparent weight of the solubilised receptor suggested a possible co-solubilisation with a G-protein. It appeared that the [^{3}H]histamine binding to bovine membranes could be inhibited by guanine nucleotides (Zweig *et al.*, 1992), confirming the results of (West *et al.*, 1990) on the coupling of the H_3 receptor to a G-protein. Furthermore, three different studies have given strong experimental evidences for the coupling of H_3 receptors to a G-protein including a PTX sensitive stimulation of [^{35}S]-GTPγS binding(Clark & Hill, 1995; Clark & Hill, 1996; Clark *et al.*, 1993).

In a study using the human gastric tumoral cell line, HGT1, Cherifi *et al.* (1992) partly purified the histamine H_3 receptor present in these cells as one single 70 kDa protein. A coupling to intracellular cAMP levels is unlikely, and an inhibition of the basal and carbachol-induced formation of phosphoinositides was detected (Cherifi *et al.*, 1992) suggesting a negative coupling of this H_3 receptor to phospholipase C (Cherifi *et al.*, 1992). The nature of the G-protein involved was not clear. Moreover, it is not known whether the inhibitory effects on IP_3 production are due to a direct effect on phospholipase C or can be explained by an inhibition of the influx of extracellular Ca^{2+}. It has been suggested that histamine can reduce neuronal Ca^{2+} entry and subsequent histamine release by inhibiting N-type Ca^{2+} channels via hyperpolarization of the neurons (Takemura *et al.*, 1989). This suggestion was supported when Yang & Hatton (1991) found that the hyperpolarization can be antagonised by the K^+- channel blockers 4-aminopyridine and triethylamine, suggesting that the H_3 receptor might be directly or indirectly linked to hyperpolarizing K^+-channels. Schlicker and colleagues (1994) showed a marked Ca^{2+} dependence of the H_3 receptor mediated inhibition of noradrenaline release which is compatible with coupling of the H_3 receptor to a G-protein. The results also suggested no coupling of the H_3 receptor to adenylyl cyclase, to ATP-dependent K^+ channels, or to (TEA-sensitive) voltage regulated K^+ channels (Schlicker *et al.*, 1994), in contrast with the suggestions made by Yang & Hatton (1991).

It can be concluded that no clear data are yet available on the second messenger involved in transducing signals from the membrane bound H_3 receptor to its effector in the intracellular compartment.

Receptor subtypes

Similarly, due to the limited data available on the biochemical characteristics of the H_3 receptor, the existence of H_3 receptor subtypes has been postulated by different research groups solely based on pharmacological data (Clapham & Kilpatrick, 1992; Leurs *et al.*, 1996; Schlicker & Kathmann, 1994; Schlicker *et al.*, 1996; Schworer *et al.*, 1994; West *et al.*, 1990). The first indications of H_3 heterogeneity were obtained from receptor binding studies (Cumming & Gjedde, 1994; Jansen *et al.*, 1994; West *et al.*, 1990) alone. As radioligand binding studies on their own cannot prove the existence of subtypes, suggestions made in these studies should be treated with caution. More recently, however, several groups have presented functional evidences for the possible existence of H_3-receptor subtypes. Most of these studies are based on comparisons of receptor affinities (obtained from binding studies) and functional data obtained from compounds which exhibit distinct pharmacological behaviours and profiles in the central nervous system versus the peripheral nervous system (Leurs *et al.*, 1996; Schlicker & Kathmann, 1994; Schlicker *et al.*, 1996; Schworer *et al.*, 1994). Nevertheless, additional studies are necessary to clarify whether the observed differences are due to differences in efficiency of receptor coupling, species differences, or H_3-receptor subtypes. Detailed information on this field is mandatory as numerous H_3 ligands are currently being investigated for their therapeutic potential in several CNS pathologies (see section 3.5. for detailed information) such as epilepsy (Yokoyama *et al.*, 1994), sleep disorders (Lin *et al.*, 1990) and Alzheimer's disease (Airksinen *et al.*, 1991; Cacabelos *et al.*, 1992; Fernandeznovoa *et al.*, 1994; Mazurkiewicz-Kwilecki & Nsonwah, 1989; Panula *et al.*, 1995).

2.2 General (patho)physiological and clinical aspects in the peripheral nervous system

The histamine H_3 receptor is involved in various physiological processes. A first 'group' of processes involving the H_3 receptor and the "waking amine" histamine is that correlated with sympathetic actions, i.e. arousal, increased locomotor activity and the suppression of food intake. Highly correlated to those physiological conditions are antinociception and anxiety, which are also partly mediated via the histamine H_3 receptor. The H_3 receptor may be involved in other processes, such as convulsions and learning. Besides the histaminergic interactions mediated via the H_3 receptor in the central nervous system, the H_3 receptor is involved in some peripheral physiological conditions as well.

The central (patho)physiological aspects of the histamine H_3-receptor will be comprehensively discussed in the next chapter. We will now focus on studies done in peripheral tissues.

Heart and cardiovascular

Histamine H_3 receptors have been shown to occur on postganglionic sympathetic neurons supplying blood vessels and the heart (Endou & Levi, 1995; Endou *et al.*, 1994) (see table 2 for further references). However, this does not appear to be a general phenomenon since, in the model of the superfused rat *vena cava*, inhibitory H_3 (unlike e.g., $GABA_B$) receptors could not be found (Schneider *et al.*, 1991).

Table 2 Occurrence of H_3 heteroreceptors in the cardiovascular system (Schlicker, 1994).

Species	*Tissue*	*Authors*
Guinea pig	Heart	(Fuder *et al.*, 1990; Luo *et al.*, 1991)
Rat	Heart	(Malinowska & Schlicker, 1993a)
Man	Saphenous vein	(Molderings *et al.*, 1992)
Pig	Vasculature of the retina	(Schlicker *et al.*, 1990)
Guinea pig	Mesenteric artery	(Ishikawa & Sperelakis, 1987)
Rat	Resistance vessels	(Malinowska & Schlicker, 1991; Malinowska & Schlicker, 1993b; Hey *et al.*, 1992b)

Histamine H_3 receptors in the cardiovascular system are not restricted to the sympathetic nerve endings but also occur in the endothelium and mediate vasorelaxation by releasing nitric oxide and prostacyclin (Ea-Kim *et al.*, 1992). Moreover, evidence for the existence of central histamine H_3 receptors modulating cardiovascular function has been presented (Imamura *et al.*, 1994; Imamura *et al.*, 1996; Mcleod *et al.*, 1994).

Airways

Histamine H_3 receptors have also been detected in lung by binding studies (Arrang *et al.*, 1987b), and various studies have investigated a functional role for these receptors in airways.

The H_3 agonist (R)-α-methylhistamine has no effect on airway smooth muscle tone *in vitro* or *in vivo*. Nor does the H_3 antagonist thioperamide influence either basal airway tone or the airway response to histamine, indicating that H_3 receptors are unlikely to be present on smooth muscle of guinea pig or human airways (Ichinose & Barnes, 1989; 1990; Ichinose *et al.*, 1989). Furthermore, inhaled (R)-α-methylhistamine has no effect

on airway function in asthmatic subjects (O'Connor & Barnes, 1990). There is no evidence for H_3 receptors in human bronchial vessels (Barnes, 1992).

(R)-α-methylhistamine has an inhibitory effect on vagus nerve-induced contraction of an innervated guinea pig tracheal tube preparation, but no effect on acetylcholine-induced contraction, (Ichinose *et al.*, 1989). The inhibitory effect is greater for vagus nerve stimulation (preganglionic), indicating that modulation occurs both at parasympathetic ganglia and postganglionically. These effects are blocked by thioperamide, but not by mepyramine or cimetidine, indicating that H_3 receptors are involved and presumably localised on parasympathetic ganglionic neurones and postganglionic cholinergic nerve terminals. Histamine, in the presence of H_1 and H_2 antagonists, has a similar inhibitory action and has no effect at low concentrations. In human bronchi *in vitro* an inhibitory effect of (R)-α-methylhistamine is seen on electrical field stimulation induced contraction, indicating a similar inhibitory effect on postganglionic cholinergic nerve, which effect is inhibited by thioperamide (Ichinose & Barnes, 1989). This demonstrates the presence of inhibitory H_3 receptors on postganglionic cholinergic nerves in human airways. Stimulation of the vagus nerve in rodents causes bronchoconstriction and airway microvascular leak which is due to the release of tachykinins, and particularly substance P, from sensory nerve endings (Barnes, 1987; Lundberg *et al.*, 1983). The release of tachykinins from airway sensory nerves may be modulated through a number of prejunctional receptors, including μ-opioid and $GABA_B$ receptors (Belvisi *et al.*, 1989; Belvisi *et al.*, 1988; Belvisi *et al.*, 1989). (R)-α-methylhistamine has an inhibitory effect on vagus nerve-induced bronchoconstriction and leakage in guinea pig airways, but no effect on the equivalent degree of bronchoconstriction or leakage induced by substance P (Ichinose & Barnes, 1989; Ichinose & Barnes, 1990; Ohkubo *et al.*, 1995). This inhibitory action of (R)-α-methylhistamine is blocked by thioperamide which indicates the presence of inhibitory H_3 receptors on airway sensory nerves (Ohkubo *et al.*, 1995). Since neurogenic inflammation may be important in asthma (Barnes, 1986), this suggest that H_3 agonists could be of therapeutic benefit in asthma and other diseases where neurogenic inflammation has been implicated (Barnes *et al.*, 1990). However, inhaled (R)-α-methylhistamine was found to have no effect on the bronchoconstriction induced by inhaled metabisulphite, which is believed to cause bronchoconstriction through activation of neural pathways (O'Connor & Barnes, 1990).

H_3 agonists inhibit the release and synthesis of histamine in central neurons (Arrang *et al.*, 1987b). It is possible that H_3 receptors may similarly inhibit synthesis and release of histamine from lung mast cells (Arrang *et al.*, 1987). Allergen induced bronchoconstriction in sensitised guinea pigs is indeed enhanced by thioperamide, but unaffected by cimetidine, whereas it is almost completely abolished by mepyramine (Ichinose & Barnes, 1990a). Since thioperamide has no effect on histamine-induced

bronchoconstriction, this strongly suggests that histamine released from pulmonary mast cells by the allergen challenge normally inhibits further release via H_3 receptors on mast cells (Barnes, 1992). Inhibiting of H_3 receptors results in increased histamine release.

Gastrointestinal tract

H_3 receptors are present in the gastrointestinal tract, intravenous infusion of the H_3 agonist (R)-α-methylhistamine, induced a dose-related inhibition of pentagastrin-stimulated gastric acid output in cats (Bado *et al.*, 1991) and of pentagastrin- and bombesin-stimulated gastric acid output in dogs (Soldani *et al.*, 1994). These effects were completely prevented by thioperamide, suggesting that they are entirely modulated via histamine H_3 receptors. Neither (R)-α-methylhistamine nor thioperamide significantly modified the increase in plasma gastrin levels induced by bombesin. Histamine H_3 receptors may represent an effective mechanism for the negative control of stimulated gastric acid secretion (Soldani *et al.*, 1994). It must be mentioned, however, that these results could not be confirmed in rats and humans (see Coruzzi *et al.*, 1992 for further references). West *et al.* (1990) already reported that different subtypes of histamine H_3 receptors (H_{3A} and H_{3B}) exist in the rat brain. Further evidence has been given by Leurs *et al.* (1996) based both on [^{125}I]iodophenpropit and N^{α}-[^{3}H]methylhistamine binding studies in rat brain cortex, and functional studies of the H_3 receptor in the guinea pig jejunum and mouse brain cortex (see section 2.1.2).

In view of the multiple efficient therapies available for the treatment of gastroduodenal ulcers a role for H_3-ligands is rather remote in these pathologies.

Besides modulating the release of gastric acid, histamine H_3 receptors are also involved in the mechanical activity of the small and the large intestine, and release of serotonin from the intestines. Various authors reported that (R)-α-methylhistamine inhibited electrically-induced contractions in guinea pig intestine, and that this effect is completely antagonised by thioperamide (Leurs *et al.*, 1991; Vollinga *et al.*, 1992). The physiological role of this effect might be related to the state of arousal in which the sympathetic nervous system is being most active. Probably also related to this mode of regulation is the the reported ability of histamine and (R)-α-methylhistamine to inhibit the release of serotonin from the porcine small intestine (Schworer *et al.*, 1992). Again, this effect was antagonised by thioperamide, indicating that the effects are mediated via histamine H_3 receptors, likely to be localised directly on the enterochromaffin cells (Schworer *et al.*, 1992).

In view of the reported inhibition of the NANC, cholinergic, and serotonergic transmission (see Leurs *et al.*, 1995 for references) H_3-receptor agonists could be seen as putative anti-diarrhoeal drugs.

2.3 Studies in human tissues

There is evidence that a histaminergic neuronal system reminiscent of that described in rodents in present in the human brain (Panula *et al.*, 1990). Using antisera against histamine, Panula *et al.* (1990) revealed a well-organised network of varicose fibres throughout the frontal and temporal cortex of adult humans. The densest area was seen in lamina I, where varicose fibres were seen to run parellel to the overlying pia matter. Hypothalamic samples obtained from autopsy brains of adult humans revealed numerous histamine-immunoreactive nerve cell bodies in the posterior basal hypothalamus in and around the tuberomammilary nucleus. Numerous neurofibrillary tangles were found in Alzheimer's disease hypothamali, concentrated in the tuberomammilary area and only a minority were histamine immunoreactive (Airksinen *et al.*, 1991). Histamine levels have also been reported to change in patients with Alzheimer's disease (Cacabelos *et al.*, 1992; Fernandeznovoa *et al.*, 1994; Mazurkiewicz-Kwilecki & Nsonwah, 1989; Panula *et al.*, 1995). Although initially conflicting results were reported, recent data has pointed towards a decrease in the levels of histamine in several specific brain areas such as the hypothalamus, temporal cortex, and hippocampus (Mazurkiewicz-Kwilecki & Nsonwah, 1989; Panula *et al.*, 1995).

In 1988, Arrang *et al.*, showed that histamine modulates its own release in human brain via stimulation of receptors with a pharmacological profile similar to that of H_3-autoreceptors controling [^{3}H]-histamine release and synthesis in the rat brain (Arrang *et al.*, 1983, 1987a, b). These findings have opened a window on the investigation of the physiological and clinical significance of H_3-receptors neuromodulatory centers for several central and peripheral functions.

In a work presented by Ichinose & Barnes (1989) it was shown that H_3-receptors are present in human airways inhibiting cholinergic transmission in the bronchi. The H_3-agonist, (R)-α–methylhistamine caused a dose-dependent inhibition of cholinergic contractile responses to electrical field stimulation in human bronchi without affecting the basal tone. This effect was prevented by thioperamide and was not affected by either H_1 or H_2 antagonists (Ichinose & Barnes, 1989).The physiological role of these H_3-receptors seems to be mainly one of defence against excess bronchoconstriction. As cholinergic reflex mechanisms are important in airways diseases, H_3-agonists could be beneficial as therapeutic agents.

Bent *et al.* (1991) suggested the existence of H_3-receptor dependent autoregulation in human adenoidal mast cells. Nevertheless, (R)-α–methylhistamine did not reverse the enhancement of spontaneous histamine release elicited by thioperamide which indicates the possibility of a non-H_3 mediated mechanism.

In a review on the modulation of neurotransmitter release *via* histamine H_3-heteroreceptors Schlicker *et al.* (1994) reported an H_3-mediated inhibitory effect of

histamine on the electrically evoked release of [^{3}H]-noreadrenaline release in human slices of cerebral cortex (Schlicker *et al.*, 1994). Although the maximum effect (25%) in the human tissue was lower than the values observed in the rat (approx. 30%), and in the mouse (50%), this effect could be important in particularly in pathological conditions where cerebral noradrenaline levels are reduced (see section 3.4.1 for further comments).

Presynaptic H_3 receptors have also been found in sympathetic nerves of the human saphenous vein (Molderings *et al.*, 1992) where they inhibit the release of noradrenaline. This effect could be of relevance for inotropic and chronotropic responses of the heart to sympathetic nerve stimulation. In fact, Endou *et al.* (1994) have shown an H_3-mediated reduction of both inotropic and chronotropic responses to field stimulation as well as a reduction of the associated noradrenaline release in isolated atria of the guinea-pig (Endou *et al.*, 1994).

3. (Patho)physiological aspects of the histamine H_3 receptor in the CNS; clinical aspects of H_3-ligands

3.1 The concept of neuromodulation

Researchers working with the brain are gradually realizing that neurons do not just transmit short-lasting, specific messages to one another. Instead, much of the signalling among neurons takes the form of *biasing* the target neuron, so that it responds in a different way to an on-off signal that may or may not be generated at a future stage. This form of communication, which in itself might not produce dramatic effects but may change the way in which a cell subsequently reacts, is know as neuromodulation (Kaczmarek & Levitan, 1987). Neuromodulation gives a neuron a recent history, a working past; without this all neurons in a certain brain area would respond in an invariant and predictable way (Fig. 3.1). Hence, we can catch a glimpse of how certain events in the brain are subtly contingent on previous ones.

The positioning of most biogenic amines in the brain is exactly what we would expect if they were working not as classical on-off transmitters but as biasing ones. As neuromodulators, these amines are in a perfect position to influence large populations of brain cells rather than transmit highly specific signals across discrete contacts. They all emanate from relatively small cell groups in the brainstem, yet, project outward into the front of the brain in a diffuse manner.

There are five distinct biogenic amines associated with the central ascending pathway and the states of arousal it generates: serotonine, dopamine, noradrenaline, acetylcholine, and histamine. The following sections will deal with the neuromodulatory action of the

presynaptic histamine H_3 receptor on the release of the amines mentioned above, including histamine itself.

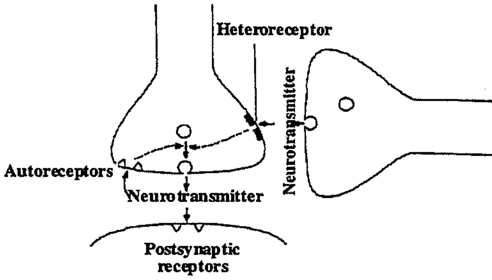


Figure 3.1 Schematic representation of presynaptic receptor control of neurotransmitter release.

3.2 Localization of the histamine H_3-receptor in the CNS

Reversible labelling of the H_3- receptor was first achieved using the highly selective agonist [^{3}H]-(R)-α-methylhistamine (Arrang *et al.*, 1987b). The binding of this radioligand is sensitive to guanyl nucleotides, strongly suggesting that, like the other histamine receptors, the H_3 receptor belongs to the superfamily of receptors coupled to G-proteins (Arrang *et al.*, 1990). N^{α}-methylhistamine has also been labelled to study the H_3 receptor (West *et al.*, 1990). Due to their discriminative characteristics between the high and low afiinity states of G-protein coupled receptors, binding data using radiolabelled agonists have been difficult to interpret. Radiolabelled antagonists are known to allow a more detailed and less ambiguous analysis of receptor binding studies. The high specific activity of the recently reported iodinated H_3 antagonists [^{125}I]-iodophenpropit (Jansen *et al.*, 1994) and [^{125}I]-iodoproxyfan (Ligneau *et al.*, 1994), makes them leading structures for the development of potent and sensitive photoaffinity label. Controversy about [^{125}I]-iodoproxyfan has increased, however. Within the concentration range expected to bind to the H_3-receptor, this radioligand is not equally displaced by H_3-agonists and antagonists in the rat brain striatum.

Yanai *et al.* (1992; 1994) recently reported on the use of S-[^{3}H]-methylthioperamide to radiolabel the H_3 receptor. Unfortunately, this study was not done in detail and questions such as high labelling of peripheral tissues where the H_3 receptors have not been previously detected remained unanswered. Several of these points were analysed in a recent report from our group in which binding studies using [^{3}H]-thioperamide to label the H_3 receptor (Alves-Rodrigues *et al.*, 1996) were performed. We showed that at concentrations known to bind mainly to the H_3 receptor, most H_3 antagonists share a

high density, low affinity, non-H_3 binding site/s, most likely due to binding to cerebral cytochrome P_{450} isoenzymes. This study also concluded that, among the radiolabelled antagonists available, [^{125}I]-iodophenpropit seems to be the most suitable compound to label the H_3 receptor, provided that agonists are used to define the nonspecific component of the binding. This conclusion is based on the observation that iodophenpropit clearly displays distinct affinities for the H_3 receptor and the non-H_3 receptor components of the binding of [^{3}H]-thioperamide to rat brain cortex (Alves-Rodrigues *et al.*, 1996).

Extensive autoradiography studies using the potent H_3 agonist [^{3}H]-(R)-α-methylhistamine (Cumming *et al.*, 1991; Pollard *et al.*, 1993) have shown high heterogeneity in the distribution of these receptors in the rat brain (see Table 3. for data on the regional distribution of histaminergic fibres and the histamine H_3 receptor in rat brain). In the cerebral cortex H_3 receptors are relatively dense and found in all areas and layers with higher abundance in rostral areas (Schwartz *et al.*, 1991). In the hippocampus they show moderate to high density with a clear abundance on the dentate gyrus (Schwartz, 1991a). The amygdaloid complex is a rich area in H_3 receptors in particular the bed nucleus of the stria terminalis which contains dense histaminergic innervation (Cumming *et al.*, 1991; Pollard *et al.*, 1993). The basal forebrain is also a rather enriched area in H_3 receptors. In this part of the brain H_3 receptors abound in the anterior olfactory nuclei, nucleus accumbens, and particularly in the dorsomedial part of the striatum (Schwartz, 1991a). They are less numerous in the globus pallidus and even less in the septum. In the thalamus and hypothalamus H_3 receptors have a moderate density which contrasts with the high density of histamine axons; they are, however, detectable at the level of the tuberomammillary nucleus where they may reside on histaminergic neurons perikarya or dendrites. In the mesencephalon, H_3 receptors are locally concentrated in the pars reticulata of the substantia nigra (Schwartz, 1991a).

In the cerebellum low densities are present in all layers, as in the spinal cord where low density is seen mainly in the external layers of the dorsal horn. This distribution of H_3 receptors does not strictly parallel that of histaminergic axons, confirming the observation of the existence of H_3 receptors in other than histaminergic neurones firstly reported by (Schlicker *et al.*, 1988). Localisation of the histamine H_3 receptor using the H_3 antagonist [^{125}I]-iodophenpropit (Jansen *et al.*, 1994) showed patterns of distribution similar to those previously described with [^{3}H]-(R)-α-methylhistamine (Pollard *et al.*, 1993).

Table 3 Regional distribution of histaminergic fibres and three histamine receptors in the brain .

	Histaminergic fibres		H_1 receptors		H_2 receptors	H_3 receptors
	Rat	Guinea Pig	Rat	Guinea Pig	Guinea Pig	Rat
Olfactory bulb		++			ND	+++
Cerebral cortex	++	++	++	++ to +++	++ to +++	+++
Hippocampus		++	++ to +++	++ to +++	+ to ++	+++
Basal ganglia	++	++	0 to +	++	+++	+++
Amygdaloid complex	++	++	++ to +++	++ to +++	ND	ND
Septum	++	+++	0 to +	+ to +++	ND	+++
Thalamus	++ to +++	++	0 to +	++ to +++	+ to ++	++
Hypothalamus	+++	+++	++ to +++	+ to +++	+ to ++	++
Superior colliculus	++	++	++	++	+++	
Inferior colliculus	++	++	++	++		
Tegmentum		++	++	++ to +++		
Medulla oblongata	+ to +++	+ to +++	+ to +++	+ to +++		
Cerebellum			0	++ to +++		
Spinal cord	+	+ to ++	++	++ to +++	ND	ND

Degree of distribution: +, low density; ++, medium density; +++, high density
ND = Not Detected; 0 = Not described (Wada, 1991).

3.3 Regulation of central histamine release

Autoregulatory mechanisms of the histaminergic activity

The histamine H_3 autoreceptor is localised at the presynaptic nerve terminals of the histaminergic neurons. Activation of the histamine H_3 autoreceptor by either histamine released from the nerve terminals, or by H_3 agonists, such as (R)-α-methylhistamine (R(α)-MeHA), will decrease the histamine synthesis and its release by the histaminergic neurons. Under physiological conditions, the H_3 receptor exerts a negative feedback on the histaminergic activity in the brain. For a comprehensive description of the histamine H_3 receptor and its functions the reader is referred to section 2.

Heteroregulatory mechanisms of the histaminergic activity

The regulation of the histaminergic activity, however, is not only dependent on histamine H_3 autoreceptors, but can also be affected by various heteroreceptors localised on the histaminergic nerve terminals.

The α_2-adrenoreceptor, when activated by noradrenaline or clonidine, was found to have an inhibiting influence on the histamine release in rat cerebral cortex (Hill & Straw, 1988) and hypothalamus (Prast *et al.*, 1990). This inhibition was reversed by phentolamine and the selective α_2-antagonist yohimbine, indicating a regulatory role in histamine release by the α_2-adrenoreceptor.

Stimulation of central muscarinic receptors potently inhibits the histaminergic activity in the brain (Gulat-Marnay *et al.*, 1989; Oishi *et al.*, 1990). Strong stimulation of central nicotinic receptors can also induce a similar effect (Oishi *et al.*, 1990). These results were confirmed by Prast (1994b). The release of endogenous histamine was found to be inhibited by carbachol, a mixed M_1, M_2, M_3 agonist, whereas the release of histamine was enhanced by atropine, a mixed M_1, M_2, M_3 antagonist. These observations lead to the conclusion that acetylcholine, released from the cholinergic neurons, also modulates the release of histamine via M_1 and/or M_3 heteroreceptors (Prast *et al.*, 1994). The fact that histamine H_3 heteroreceptors modulate acetylcholine release and that muscarinic M_1 receptors modulate histamine release, suggests a crosstalk communication between presynaptic muscarinic and histaminergic receptors. In particular in the hippocampus, this crosstalk could be of relevance in learning and memory processes.

κ-opioid receptors were also found on presynaptic histaminergic nerve terminals (Gulat-Marnay *et al.*, 1990). Activation of these hetero κ-opioid receptors attenuates the histamine release in the brain, which may enhance the sedative actions of κ-opioid agonists. It must be noted, however, that under basal conditions these κ-opioid receptors are not tonically activated by endogenous dynorphin peptides, and, therefore, do not play a role under standard physiological conditions.

3.3.1 (Patho)physiological implications

Up to now, no H_3-ligands have been introduced in therapy, but intensive research by several groups has suggested several putative applications. In fact, a new brain-penetrating, non-thiourea H_3-antagonist, GT-2016 (see chapter 3.5.) has recently entered a phase I clinical trial (Tedford *et al.*, 1995).

Sleep / wakefulness

Injection of the selective H_3 receptor agonist (R)-α-methylhistamine into the premammillary area, increased slow-wave sleep and decreased wakefulness and REM sleep (Monti *et al.*, 1991). When (R)-α-methylhistamine was administered intraperitoneal (i.p.), no significant changes in sleep variables could be detected. This could be due to

the fact that systematically (i.p.) administered (R)-α-methylhistamine does not enter the brain of the rat in concentrations sufficient to produce significant changes of sleep and wakefulness (Monti *et al.*, 1991).

Slow-wave sleep induced by (R)-α-methylhistamine given i.c.v. was prevented by thioperamide, which is consistent with the histamine releasing effect of thioperamide. Pyrilamine, an H_1-receptor antagonist was found to block the effects of thioperamide (Lin *et al.*, 1990), suggesting that the arousing effect of the H_3 antagonist is related to activation of central H_1 receptors what could also explain the sedative effects of H_1-receptor antagonists (Rose *et al.*, 1982; Terlaak *et al.*, 1994). It must be noted, however, that inhibition of e.g. serotonin and noradrenaline activity by the H_3 agonist (see 3.4.3. and 3.4.1, respectively) may be partly responsible for the slow-wave sleep increase (Monti *et al.*, 1991).

Locomotor activity and anxiety

Thioperamide has been reported to increase locomotor activity of W/W^v mice with a concomitant decrease in their whole-brain histamine content (Sakai *et al.*, 1991), suggesting that thioperamide activates the histaminergic neuronal system and causes hyperactivity via histamine released from the terminals (Sakai *et al.*, 1991). The increase in the locomotor activity by thioperamide was blocked by i.p. pretreatment with (R)-α-methylhistamine, an H_3 agonist; pyrilamine, an H_1 antagonist; or zolantidine, an H_2 antagonist (Sakai *et al.*, 1991); thus, confirming that the increased locomotor activity is likely mediated via H_1/H_2 receptors. From these results it is also evident that α-fluoromethylhistamine, a histidine decarboxylase inhibitor, can decrease locomotor activity. (R)-α-methylhistamine however, was not able to decrease the locomotor activity by itself but only in combination with α-FMH. The mechanism of this interaction remains unclear (Sakai *et al.*, 1993). Concerning the role of thioperamide in locomotor activity, Clapham & Kilpatrick (1994) reported a role of the histamine H_3 receptor in modulating stimulant-induced locomotor activity in the mouse. Intraperitoneal (i.p.) administration of thioperamide inhibited, in a dose-dependent manner, the increase in locomotor activity induced by amphetamine. This inhibitory response evoked by thioperamide was reversed by the H_3 agonist (R)-α-methylhistamine. Clapham & Kilpatrick reported that neither thioperamide nor (R)-α-methylhistamine affected spontaneous locomotor activity. These results contrast with those discussed from Sakai *et al.* (1991, 1993) but confirm those of Monti (1991).

Another condition closely related to the activated state of arousal, is that of anxiety. Imaizumi *et al.* (1993) reported that thioperamide induces the release of endogenous neuronal histamine, which in turn stimulates both H_1 and H_2 receptors. The stimulation

of histamine H_1 receptors may mediate the anxiety, while H_2 receptors may play a role in masking the anxiogenic effect (Imaizumi & Onodera, 1993).

Circadian rhythms

Sakai *et al.* (1992a) reported a direct correlation between cerebral histamine content and locomotor activity. The hypothesis that histamine is a 'waking amine' was supported by Monti (1993) who reported that the histaminergic activity in rats was significantly increased during the awake or light periods, while the histamine levels reached a minimum during the dark periods. It was also shown that administration of thioperamide increased wakefulness, while (R)-α-methylhistamine, mepyramine and the HDC inhibitor α-fluoromethylhistidine produce the opposite effects (Monti, 1993). Besides decreasing wakefulness, α-fluoromethylhistidine also induces food intake, while this effect is antagonised by thioperamide (Sakata *et al.*, 1991; Sakata *et al.*, 1991). Furthermore, glucodeprivation was found to increase turnover, synthesis and release of histamine in the hypothalamus (Sakata *et al.*, 1994), suggesting that the histaminergic system may play a role in maintaining metabolic energy balance in rats. This observation might also be the basis for the reported side-effects of weight gain subsequent to H_1-antagonist therapy. In rats, the application of thioperamide results in a decrease in food intake (Doi *et al.*, 1994; Sakata *et al.*, 1991; Sakata *et al.*, 1991), suggesting a putative application of H_3-receptor antagonists in weight disorders. Mochizuki *et al.* (1992) found that neuronal histamine from the anterior hypothalamic area is released in a circadian fashion, suggesting that the central histaminergic system is related to the circadian rhythm of rats (Mochizuki *et al.*, 1992). These results were confirmed by Doi *et al.* (1994) who reported that a sustained infusion of α-fluoromethylhistamine into the rat third cerebral ventricle disrupted light-dark cycles of feeding, drinking, and ambulatory behaviour. Overall, it can be concluded from these data that histamine plays a major role in the control of the circadian rhythm, regulating the overall state of activity in a sympathetic fashion.

Neuroendocrine regulation

Histamine has been shown to be involved in the central regulation of pituitary hormone secretion (Knigge & Warberg, 1991). I.c.v. administered histamine stimulates the secretion of ACTH and β-endorphin via activation of central histamine H_1 and H_2 receptors (Knigge *et al.*, 1988). Furthermore, Knigge *et al.* have shown that blockade of central H_1 and H_2 receptors attenuated or prevented the release of ACTH or β-endorphin to restrain or ether stress (Knigge *et al.*, 1990), indicating that histaminergic hypothalamic neurons are involved in the mediation of the stress-induced release of the pro-opiomelacortin (POMC)-derived peptides. Moreover, in a paper published in 1992 this group showed that the stimulatory effect of histamine and restraint stress on the

release of POMC-derived peptides from the anterior pituitary involves corticotropin releasing hormone (CRH) and arginine-vasopressin (AVP). In a more recent work, Kjær *et al.* (1994) reported that dehydration-induced AVP release involves activation of hypothalamic histaminergic neurons, resulting in the release of histamine, indicating that histamine serves as a physiological regulator of the AVP release that subsequently is involved in the regulation of both anterior pituitary hormone secretion and water metabolism (Kjaer *et al.*, 1994).

In addition, it was reported that histamine inhibits the basal and stimulated growth hormone (GH) secretion in the rat (Netti *et al.*, 1991). However, Netti *et al.* (1991) later reported that acute systemic administration, of the selective histamine H_3 agonist (R)-α-methylhistamine and the H_3 antagonist thioperamide at doses known to act on the H_3 in the CNS (Garbarg *et al.*, 1989), did not significantly affect basal secretion of either prolactin or GH. The lack of effect of H_3 drugs on basal prolactin and GH suggests that endogenous histamine has only a minor role in the tonic secretion of these hormones (Netti *et al.*, 1991). This minor role can be shown by an indirect stimulant-induced hormone secretion. Indeed, thioperamide significantly enhances the morphine-induced prolactin release, while it inhibits growth hormone release (Netti *et al.*, 1991). The growth hormone release inhibition was reversed by (R)-α-methylhistamine, and these results indicate an effect via presynaptic histamine H_3 receptors. In contrast to the results just described, Navarro *et al.* (1993) reported that (R)-α-methylhistamine, a histamine H_3 agonist, evokes prolactin release by activation of postsynaptic H_3 receptors. This stimulatory effect of postsynaptic histamine H_3 heteroreceptors was found when the agonist was injected into the lateral ventricle of the rat brain. Its stimulatory action was prevented by thioperamide (Navarro *et al.*, 1993).

This same effect of stimulatory postsynaptic H_3 receptors, was also found earlier by Schwartz *et al.* (1991) for enkephalin release in the striatum. These results are illustrative of the fact that different parts of the brain can be controlled in different ways, depending on the nature and origin of the modulatory signals.

Cognitive functions

Besides playing a regulatory role in the waking state, the histaminergic system has also been found to be involved in memory and learning processes. In rodents, posttraining memory was facilitated by histamine administration (De Almeida & Izquierdo, 1988; De Almeida & Izquierdo, 1986). Furthermore, it was demonstrated that endogenous histamine from mast cells is not involved in memory facilitating processes, but rather histamine released from histaminergic nerve terminals (De Almeida & Izquierdo, 1988). Although showing sometimes conflicting results, the involvement of the histaminergic neuronal system in cognitive functions was further supported by Smith *et al.* (1994) (Smith *et al.*, 1994) and Meguro *et al.* (1995) by demonstrating that (R)-α-

methylhistamine and thioperamide are indeed effective in modulating cognitive processes in the brain. Miyazaki *et al.* (1995a) showed that, via histamine H_1 receptors, histamine elicited an ameliorating effect on the scopolamine-induced learning deficit, further supporting the concept that histamine may play an important role in learning and memory. The mechanism underlying this effect is not quite clear. It was suggested that thioperamide induces the release of neuronal histamine, which, in turn, stimulates both histamine H_1 and H_2 receptors. Especially, stimulation of central histamine H_1 receptors leads to the ameliorating effect on memory loss, while central histamine H_2 receptors may counteract this effect (Miyazaki *et al.*, 1995).

In correlation to these cognitive functions, it was found that brain histamine and histidine levels in Alzheimer's patients are significantly decreased in certain brain areas, suggesting a disturbed brain histamine regulation in this disease (Mazurkiewicz-Kwilecki & Nsonwah, 1989; Panula *et al.*, 1995).

Depression

In a recent study, antidepressant-like effects were reported for substances which enhance histamine brain levels in mice. The H_3-receptor antagonist thioperamide reduced the duration of immobility in a Porsolt forced swim test. This effect was reversed by (R)-α-methylhistamine which, by itself, did not have any effect (Lamberti *et al.*, 1996) on the test. Metoprine and L-histidine mimicked the effect of thioperamide further supporting the role of the histaminergic neuronal system in the control of depressive states.

Schizophrenia

An opposite type of disturbed brain histamine regulation is found in patients with chronic schizophrenia. In these patients the histaminergic activity has been significantly increased as shown by an increased amount of histamine metabolites in their cerebrospinal fluid (Prell *et al.*, 1995). It is possible that the histaminergic system does not control these pathologies, but instead is being controlled by them. Reports by different groups on the histamine H_2-receptor antagonist famotidine induced reduction of negative symptoms in schizophrenic patients (Deutsch *et al.*, 1993; Kaminsky *et al.*, 1990) further supports a possible role of this amine in this psychotic disease. Restoration of the original histaminergic activity using H_3-agonists might contribute to a therapeutic approach of these diseases.

Epilepsy

A possible role of histamine in epilepic seizures has often been suggested. Tuomisto and coworkers (1987) found a general anticonvulsant effect provoked by metropine-induced increase in brain histamine. Moreover, mice treated with a histamine synthesis inhibitor (α-fluoromethylhistidine) showed a higher susceptibility to seizures induced by

electroshock. Supporting this hypothesis are the abormally low levels of histamine found in the brain stem of genetically epilepsy-prone rats when compared with resistant control rats (Onodera *et al.*, 1992). In epileptic patients higher H_1-densities in the electrical focal of the temporal neocortex were found when using the PET probe [^{11}C]-doxepin (Iinuma *et al.*, 1993). This points to a functional upregulation of H_1-receptors to counteract the low levels of histamine in epileptic brains. Recent studies have also shown that the H_3-receptor antagonists thioperamide, clobenpropit, or i.c.v. administered histamine significantly decreased the duration of electrically induced convulsions through activation of the H_1 receptor (Yokoyama *et al.*, 1993; Yokoyama *et al.*, 1993; Yokoyama *et al.*, 1994) while this effect could be antagonised by (R)-α-methylhistamine. When applied centrally, clobenpropit is approximately 10-fold more potent than thioperamide, which makes it a promising research tool to investigate these type of neuropathologies. A contrasting study was presented by Sturman *et al.* (1994); in this study the H_3-antagonists thioperamide and burimamide potentiated the severity of clonic convulsions induced by picrotoxin. It should be noticed, however, that thioperamide was subcutaneously administered, which makes uncertain the amount of compound that actually reached the CNS. Burimamide effects could be due to H_2-receptor antagonism.

Taken together the main stream of results indicate a protective role for neuronal histamine in seizures.

Motion sickness and vertigo

Another indication for the usage of an H_3 antagonist is related to motion-sickness and vertigo. Betahistine, a mixed H_1 agonist / H_3 antagonist of moderate potency (Arrang *et al.*, 1985), is currently prescribed for the symptomatic treatment of vestibular-related syndromes, and it is suggested that H_3 antagonists, such as thioperamide, are powerful tools for symptomatic treatment of peripheral vertigo and central vestibular disorders (Yabe *et al.*, 1993).

Antinociception and sedation

To some extent antinociception can be considered as a sympathetic physiological reaction. Thioperamide, at a dose increasing the histaminergic turnover, also elicites an antinociceptive effect (Malmberg-Aiello *et al.*, 1994). This antinociceptive effect can be completely prevented by pretreatment with (R)-α-methylhistamine, which in its own has a hyperalgesic effect. The antinociceptive effect was also induced by i.p. administration of L-histidine, and i.p. or i.c.v. administration of metoprine (Malmberg-Aiello *et al.*, 1994). These reults indicate that the antinociceptive activation of histamine may take place on a postsynaptic site, and that its hyperalgesic effect occurs with low doses acting on the presynaptic receptor. Furthermore, it has been shown by Barke & Hough (1994) that morphine acts on histaminergic nerve terminals, to increase extracellular histamine release

in the rat periaqueductal gray (PAG). It is suggested that the histaminergic nerve terminals in the PAG are under tonic GABAergic inhibition and are thus indirectly excited by morphine. Since thioperamide has the same ability to increase the histamine release in the rat PAG, it is suggested that thioperamide might be a potential analgesic agent (Barke & Hough, 1994).

Migraine

Already in the 60's parenteral infusion of histamine has been found to cause pulsatile headache in both normal subjects and migraine patients (Sicuteri, 1963). In general, the latter group reacts to lower concentrations of histamine. On the other hand, infusion of either serotonin or noradrenaline brings relief to migraine pain (Anthony, 1981). As antihistamines (H_1-receptor antagonists) are also known to inhibit the reuptake of serotonin and noradrenaline in rat brain synaptosomes (Young *et al.*, 1988), one could speculate that administration of H_1 receptor antagonists and/or reduction of brain histamine levels using H_3 receptor agonists could be beneficial in attenuating migraine pains. Although clinical responses to H_1 antagonists therapy alone have been rather disappointing in ameliorating vascular headaches (Mansfield, 1990), Matsubara *et al.* (1992) (Matsubara *et al.*, 1992) showed that i.v. administration of (R)-α-methylhistamine inhibited electrically stimulated non-adrenergic, non-cholinergic (as well as capsaicin induced) neurogenic plasma extravasion in the rat dura mater. The similarity between this effect and that of the previously reported 5-HT_{1B}/5-HT_{1D} agonists, commonly used in the treatment of acute migraine (Buzzi *et al.*, 1992), raised the possibility of the use of H_3-receptor agonists in the treatment of migraine.

Food intake and satiety

Bombesin (BN) and its mammalian homologue, gastrin-releasing peptide (GRP), are potent satiety agents and have been shown to be involved in the physiological regulation of food-intake. There are indications for interaction with the histaminergic system, and it has been suggested that BN may mediate its satiety effects through activation of the histaminergic system. Merali and Banks (1994) reported that BN reduced food-intake by > 50% relative to the control condition; this suppression was blocked by prior treatment with the histamine H_3 receptor agonist (R)-α-methylhistamine. When (R)-α-methylhistamine was administered alone, it failed to significantly affect food intake. The specificity of this effect was further supported by the demonstration that another H_3 agonist, imetit, was also able to block the feeding-suppressant effects of BN. Furthermore, thioperamide blocked the effect of imetit (Merali & Banks, 1994).

3.4 The histamine H_3 heteroreceptor

Central H_3 heteroreceptors have been shown to be present on various types of neurons, particularly on monoaminergic neurons (see Table 4). It has been suggested by Schlicker *et al.* (1992; 1994) that the α_2-adrenoreceptors and the H_3 heteroreceptors at the noradrenergic nerve endings in the brain of rat and mouse interact with each other. These same suggestions were made for the interaction of H_3 heteroreceptors and dopamine autoreceptors on the dopaminergic nerve endings in the mouse striatum (Schlicker *et al.*, 1993). The physiological role of this wide-spread ocurrence of the histamine H_3 heteroreceptor remains unclear, but does support earlier suggestions that the histaminergic neuronal system might be a regulatory centre for whole-brain activity. In order to analyse this 'overall function', we will examine to what extent histamine modulates the release of the various monoaminergic neurotransmitters, and what the clinical interests of these effects may be.

It should be noticed that most of the studies concerning the role of histamine H_3 heteroreceptors in regulating the release of other neurotransmitters were performed in rodents. To assess the relevance of these effects as clinical and therapeutic targets, it is crucial to investigate their ocurrence in human brain.

Table 4 Occurrence of H_3 heteroreceptors in the central nervous system. The presynaptic location of the H_3 receptors has been proven for the serotonergic and noradrenergic neurones of the rat and mouse brain cortex and the dopaminergic neurones of the mouse striatum (Schlicker *et al.*, 1994).

Type of neuron	*Species*	*Brain region*	*Authors*
Noradrenergic	Man, rabbit, guinea pig	Cerebral cortex	Schlicker *et al.* (1994)
	Rat	Cerebral cortex	Schlicker *et al.* (1989), Smits (1991)
		Hypothalamus	Smits & Mulder (1991),
	Mouse	Cerebral cortex	Schlicker *et al.* (1992)
Dopaminergic	Mouse	Striatum	Schlicker *et al.* (1993)
Serotonergic	Rat	Cerebral cortex	Schlicker *et al.*(1988), Fink *et al.* (1990), Smits & Mulder (1991)
		Striatum, Hypothalamus	Smits & Mulder (1991)
Cholinergic	Rat	Entorhinal cortex	Clapham & Kilpatrick (1992)
		Ventral striatum	Prast *et al.* (1993)

3.4.1 Regulation of noradrenaline release

Histamine was found to have an inhibitory effect on the release of noradrenaline in the rat brain cortex (Schlicker *et al.*, 1989; Smits & a.h., 1991), and the mouse brain cortex (Schlicker *et al.*, 1992). It has been confirmed that presynaptic histamine H_3 heteroreceptors are involved, and that these heteroreceptors are indeed localised on the noradrenergic nerve terminals (Fink *et al.*, 1994). Whether these receptors are activated by released endogenous histamin , under physiological conditions, and play a role in interneuronal communication remains unclear. The relatively high affinity of histamine for the H_3 receptor, however, strongly points to a tonic action at these heteroreceptors. As noted above, α_2-autoreceptors and the H_3-heteroreceptors at the noradrenergic nerve endings in the brain of the mouse and the rat interact with each other (Schlicker *et al.*, 1992). The underlying mechanism of this crosstalk, however, remains unclear. Activation of the α_2-autoreceptors decreases, whereas blockade of the activated (but not of the non-activated) α_2-autoreceptors increases the inhibitory effect of histamine (Schlicker *et al.*, 1994). If assumed that the physiological role of heteroreceptors is minor when the level of endogenous noradrenaline in the biophase is high, but increases under pathological conditions when noradrenaline release from the axon terminals (Schlicker *et al.*, 1992) decreases, it can be speculated that interactions between the presynaptic α_2 autoreceptor and the presynaptic H_3 heteroreceptor on the noradrenergic nerve terminals possess (patho)physiological significance.

Furthermore, stimulatory NMDA receptors were also found to be located presynaptically on the noradrenergic terminals in the rat brain cortex (Fink *et al.*, 1989; Fink *et al.*, 1990), and the NMDA-evoked noradrenaline release in the cerebral cortex can also be modulated by inhibitory H_3 receptors. The functional interaction between H_3 and NMDA receptors may be involved in an inhibitory histaminergic influence on NMDA receptor-mediated responses (see chapter 3.4.4). Therefore, the histaminergic neuronal system in the rat cerebral cortex may modulate physiological functions in which the NMDA receptor system is involved, such as modulation of noradrenaline release (Fink *et al.*, 1994).

Schizophrenia

Noradrenaline interacts with dopaminergic systems in various regions of the brain. Tassin (1992) reviewed the evidence for this interaction in the cortex and pointed out that a reciprocal control exists between dopaminergic D_1 receptors and adrenoreceptors. Many antipsychotic drugs are antagonists at adrenoreceptors, although the α_1 blockade shared by several of these drugs (Richelson & Nelson, 1984) is considered to be responsible for some of the unpleasant side effects. However, Tassin (1992) concludes that α_1 blockade, by disinhibiting dopaminergic transmission via D_1 receptors, may play a role in the

antipsychotic response, particularly in diminishing positive symptoms in schizophrenia (Tassin, 1992). Direct clinical evidence in support of such an α_1-mediated antipsychotic action is lacking, however.

Clozapine, alone among antipsychotics, possesses an effective α_2 antagonism (Richelson & Nelson, 1984), which is probably responsible for its ability to increase plasma noradrenaline. A recent study has shown that treatment of schizophrenia with a typical antipsychotic drug, fluphenazine, and with the α_2 antagonist, idazoxan, leads to an improvement on both positive and negative schizophrenic symptoms (Litman *et al.*, 1993). The improvement was similar to the response induced by clozapine in these patients, implying that α_2 antagonism may well contribute to the clinical efficacy of this drug. Litman *et al.* (1993) suggest that this effect may be related to an enhancement of noradrenergic transmission resulting from blockade of presynaptic α_2 autoreceptors.

Depression

Concerning the involvement of noradrenaline in the treatment of depression, it was found that under depressive conditions, the activity of the noradrenergic system was reduced (Blier & de Montigny, 1994). It was also reported, however, that depletion of noradrenaline in healthy individuals does not induce clinically significant depressive symptomatology (Blier & de Montigny, 1994). This result suggests that this monoamine neurotransmitter is not essential to maintain normal mood (Blier & de Montigny, 1994). An involvement of the noradrenergic system with other systems such as the dopaminergic and serotonergic systems, is known to be present in depression (Blier & de Montigny, 1994).

Sleep

A completely different pathology in which noradrenaline has been suggested to play a role, is that of sleep and wakefulness. It has been shown that the noradrenergic system plays a role in the waking EEG (see for references Monti *et al.*, 1991). Inhibiting the release of noradrenaline by (R)-α-methylhistamine, acting on the histamine H_3 heteroreceptors at the noradrenergic nerve endings, has been suggested to be partly responsible for the slow-wave sleep increase in the rat (Monti *et al.*, 1991).

Cognitive functions

Little is known about the relationshipp between catecholamines (CA) and cognitive performances. Significant cell loss in *locus coeruleus*, the major source of cortical and hippocampal noradrenaline, occurs with advancing age in brain from humans and mice (see McEntee & Crook (1990) for references). Impaired regulation of brain adrenergic receptors may occur as a consequence of ageing and age-related decreases in the density of catecholamines receptors are described in human and animal brains (McEntee &

Crook, 1990). Arnsten & Goldman-Rakic (1985) tested a number of drugs that affect CA activity for their influence on spatial memory in aged rats. The most effective of these drugs was clonidine which strikingly improved performance in all the animals tested (Arnsten & Goldman-Rakic, 1985). These studies also showed that the greater the loss of noradrenaline by toxin-induced depletion, the lower the amount of drug required for optimal mnemonic improvement. Taken together, these findings prompted Arnsten & Goldman-Rakic to hypothesize that the beneficial effect of clonidine on memory in aged monkeys are due to denervation supersensitivity of prefrontal and hippocampal α_2 receptors. This view receives added support from recent studies using other α_2 agonists such as guanfacine (Arnsten *et al.*, 1988).

In a simple mechanistic fashion, it can be suggested that H_3-receptor agonists, decreasing the noradrenaline release, could be clinically interesting in the treatment of psychotic disorders or as sleep and memory enhancers, whereas H_3 antagonists, enhancing noradrenaline release, could be useful as antidepressant agents.

3.4.2 Regulation of dopamine release

Histamine was found to have an inhibitory effect on the release of dopamine in the mouse striatum (Schlicker *et al.*, 1993). This inhibition was shown to be mediated through presynaptic histamine H_3 receptors. Schlicker *et al.* (1993) also suggest that the H_3 heteroreceptors and the dopamine autoreceptors on the dopaminergic nerve terminals are functionally coupled (at the level of the receptors themselves or at the stage of the post-receptor transduction mechanism). This finding is comparable to the coupling between the H_3 hetero- and the α_2 autoreceptors on the noradrenergic nerve terminals of the rat and mouse brain cortex (Schlicker *et al.*, 1992). Histamine may have a marked inhibitory influence on dopamine release when the activity of histaminergic neurons is high and, simultaneously, that of dopaminergic neurons is rather low, leading to a relatively low level of autoreceptor activation by endogenous dopamine (Schlicker *et al.*, 1993).

The dopaminergic neuron system in the brain is found to be involved in two major pathologies. In psychotic disorders, e.g. schizophrenia, the dopaminergic system is found to be hyperactive (Reynolds & Czudek, 1995), while in Parkinson's disease the dopaminergic neuronal system in the *substantia nigra* is degenerated, so that the dopamine levels in the brain are too low. In Parkinsonism, the dopamine levels have lost their natural balance with acetylcholine levels; this imbalance leads to an overstimulation of the extrapyrimidal system which causes tremor, bradykinesia, rigidity, and postural instability (Cutson *et al.*, 1995). Dopamine is not independently involved in these two

pathologies, in schizophrenia therapy dopamine antagonists, may, as side effects, lead to Parkinson's disease-like symptoms.

Schizophrenia

Clozapine, the first of the so-called atypical antipsychotic drugs developed, is a tricyclic dibenzodiazepine (Figure 3.1). This drug is particularly useful in the treatment of neuroleptic-resistant schizophrenia and has been shown to significantly improve both positive and negative symptoms in patients (Kurz *et al.*, 1995).

H

H_3C—N N— NH

N—

Cl

Figure 3.1 The chemical structure of clozapine

Although clozapine also demonstrates antagonism at the dopamine D_2 receptor, its affinity for this receptor is relatively weak compared with the classical antipsychotic agents (Brucke *et al.*, 1992), and its *in vivo* and therapeutic levels of occupancy of the receptor in humans is in the range of 20 to 67% (Farde *et al.*, 1994; Nordstrom *et al.*, 1993). This finding may account for the low incidence of extrapyramidal symptoms associated with clozapine in contrast to other neuroleptics (Farde *et al.*, 1994). In contrast, clozapine appears to have a relatively high dopamine D_1 receptor occupancy (33 to 59%) (Farde *et al.*, 1994) compared with other antipsychotic agents (thioridazine 30%; flupenthixol 16 to 44%; haloperidol 3%; sulpiride 0%) (Van Tol *et al.*, 1991), and a high affinity for the dopamine D_4 receptor (about 10 fold higher than that for the pharmacologically similar D_2 and D_3 receptors) (Van Tol *et al.*, 1991). Clozapine also binds to dopamine D_5 receptors (Sunahara *et al.*, 1991).

The atypical aspects of clozapine activity may also be associated with its affinity for several other neurotransmitter receptors. The relative lack of extrapyramidal symptoms associated with clozapine may, in part, be explained by its antimuscarinic effect (Wagstaff & Bryson, 1995).

As has been found for sulpiride, the neostriatum dopaminergic system is relatively unaffected by clozapine (Brucke *et al.*, 1992). Catalepsy is not induced by clozapine in rodents, and amphetamine- or apomorphine-induced stereotypy is unaffected by the drug.

Instead, amphetamine- or apomorphine-induced locomotion is inhibited by acute administration of clozapine, and long term administration supersensitises those behaviors mediated via mesolimbic dopaminergic pathways (dopamine-induced locomotion) but not those mediated via neostriatal systems (dopamine-induced stereotypy) (Fitton & A.C., 1990). It was also found that, in the rat, this amphetamine-induced locomotion can be attenuated by thioperamide possibly via an interaction with presynaptic histamine H_3 heteroreceptors located at the nerve terminals of the dopaminergic neuronal system (Clapham & Kilpatrick, 1994). Interestingly, clozapine was also found to act as an antagonist at histamine H_3 receptors, blocking the H_3 receptor-mediated inhibition of electrically evoked release of serotonin (Alves Rodrigues *et al.*, 1995) and noradrenaline (Kathmann *et al.*, 1994) in the rat cerebral cortex. Recent findings show that if binding to the H_3 receptor does account for the clinical efficacy of clozapine, then it is not through its major metabolites clozapine-N-oxide and desmethylclozapine, as both compounds showed significantly lower affinity for the H_3 receptor than clozapine (Alves-Rodrigues *et al.*, 1996). These observations might support the concept of an increase in histamine levels during neuroleptic therapy and, therefore, changes in histamine levels are probably not a cause but a consequence of the development of psychotic disorders such as schizophrenia.

Parkinson's disease

Parkinson's disease is primarily characterised by disorders of movement resulting from a deficiency of dopamine in motor control pathways of the central nervous system. There is a degeneration of neurons in the substantia nigra that produce dopamine. The lack of the neurotransmitter dopamine in the basal ganglia results in Parkinson's disease. It has been shown that 6-OH-dopamine-induced denervation of dopaminergic neurons results in a marked increase in the density of histamine H_3 receptors in the striatum and substantia nigra of the rat (Ryu *et al.*, 1994). These results suggest that H_3 receptors in these areas are under tonic dopaminergic influence. Since this dopaminergic influence is diminished in Parkinson's disease, administration of histamine H_3-agonists, to mimick the dopaminergic influence, could be helpful in ameliorating Parkinsonian symptoms.

Anticholinergic drugs also attenuate the symptoms of Parkinson's disease by rectifying the imbalance between dopamine and acetylcholine in the striatum. Anticholinergics were the first drugs used to treat patients with Parkinson's disease, and the symptom most improved by these agents is tremor (see (Cutson *et al.*, 1995) for references). Administration of H_3 agonists could also be useful via inhibition of acetylcholine release in striatal areas. Nonetheless, the effect could only be advantageous when dopaminergic transmission is compensated by other therapeutic agents.

Another way to comprise the increase of the available dopamine levels in the brain is the administration of a selective MAO-B inhibitor, such as selegiline, which is one of the

enzymes that catabolise dopamine in the brain. In this sîtuation histamine release is also inhibited by activation of H_3 autoreceptors due to accumulation of histamine (see figure 1.3).

If the H_3 receptor modulation of the dopaminergic activity is also found in humans, reducing the H_3 mediated inhibition of dopamine release by using H_3-antagonists could be therapeutic benefits in Parkinson's disease while the opposite could help schizophrenic patients.

3.4.3 Regulation of serotonin release

In 1988 Schlicker *et al.* demonstrated that the electrically evoked release of serotonin (5-hydroxytryptamine, 5-HT) from serotonergic nerve terminals in the rat brain cortex is inhibited by histamine via histamine H_3 heteroreceptors. These results were later confirmed by Smits & Mulder (1991) (Smits, 1991) in slices of rat brain cortex. It was also found that this action was mimicked by the histamine H_3 receptor agonists (R)-α-methylhistamine and N^{α}-methylhistamine, while this inhibitory effect on serotonin release was reversed by the H_3 receptor antagonist thioperamide (Fink *et al.*, 1990; Schlicker *et al.*, 1991). These results provide evidence that endogenous histamine inhibits serotonin release in the rat brain cortex via presynaptic histamine H_3 heteroreceptors.

Like other monoaminergic transmitter systems, the serotonergic neuronal system is involved in various processes in the brain. An involvement in psychotic disorders is suggested, as well as a role in anxiety and depression (Blier & de Montigny, 1994).

Modulation of the serotonergic activity via histamine H_3 heteroreceptors could be a helpful complementary therapeutic approach in these disorders.

Schizophrenia

Along with its relatively lower blockade of dopamine D_2 receptors, blockade of 5-HT_2 receptors by clozapine may be associated, with the decreased incidence of extrapyramidal symptoms (see Wagstaff & Bryson, 1995 for references) and with the improvement of negative symptoms in schizophrenia. High 5-HT_2 receptor occupancy was also recently found in patients treated with the 5-HT_2 blocker risperidone. It is still unclear whether the high 5-HT_2 occupancy contributes to the efficacy of atypical antipsychotics. All such drugs still have significant affinity for dopamine D_2 receptors (Sedvall & Farde, 1995).

Learning and memory

Unfortunately, animal data clearly show that the 5-HT_{1A} receptor partial agonist buspirone also disrupts acquisition avoidance learning and retention, leading to the question of a link between anxiety, memory, and learning. Indeed, in contrast with anxiolytics, anxiogenic drugs, notably β-carbolines, promote learning capacity in rats. As

the same limbic structures (amygdala, hippocampus and septum) are implicated in both anxiety and memory, it is probably illusory to expect to find anxiolytics with no (negative) effects on cognition in humans (see Hamon, 1994 for references). Since thioperamide has an anxiogenic effect, as has been shown by Imaizumi & Onodera (1993), it is possible that the learning promoting characteristics of thioperamide may be related to its increase on serotonin release or vice-versa.

Depression

Since most successful antidepressants are serotonin reuptake inhibitors (e.g. Prozac; (Blier & de Montigny, 1994), it seems likey that the most important appliance of H3 ligands in relation to the H3 receptor modulation of serotonin release is in this field. Histamine H3-receptor antagonists may play a similar role in the treatment of depression by enhancing serotonin release and, consequently, increasing serotonin levels at the synaptic cleft.

3.4.4 Regulation of acetylcholine release. Implications in cognitive functions

In 1992 Clapham & Kilpatrick established that the release of acetylcholine (ACh) from the cholinergic neuron system in the rat enthorinal cortex is modulated via histamine H3 heteroreceptors. The inhibitory effect of (R)-α-methylhistamine on this release was antagonised by thioperamide (Clapham & Kilpatrick, 1992). These observations were supported by Mochizuki *et al.* (1994) in the rat hippocampus, but the effects were weak and relatively short-lasting compared to direct histamine stimulation (Itoh *et al.*, 1992; Mochizuki *et al.*, 1991). It is proposed that participation of presynaptic H3 receptors in the tonic regulation of acetylcholine release may be minor, at least in the hippocampus *in vivo*, where the histaminergic innervation is at a low level (Mochizuki *et al.*, 1994). Nevertheless, the importance of this action may dramaticaly increase in (patho)physiological conditions where acetylcholine levels in the hippocampus are reduced such as in senile dementia of the Alzheimer type (SDAT) (Amenta *et al.*, 1995).

Prast and colleagues (1994) reported that the release of acetylcholine in the ventral striatum is also enhanced by thioperamide and the histamine H_1 agonist 2-thiazolyl-ethylamine, while (R)-α-methylhistamine remained ineffective. The authors suggest that histamine exerts modulatory influences on striatal cholinergic neurons by stimulation of H_1 receptors, and thioperamide might enhance the release of endogenous histamine which in turn modulates acetylcholine release via H_1 receptors (Prast *et al.*, 1994). Furthermore, *in vivo* modulation of cerebral cortical ACh release by the H3 receptor was recently described (Blandina *et al.*, 1996). It was also reported in this work that this process was not mediated by presynaptically H3-receptors located on cholinergic

terminals. The antagonistic effect of the GABA antagonist, bicuculline, suggests an indirect effect of the H_3 receptor on cortical ACh release through activation of GABA release.

Hippocampal memory formation

Biochemical and pharmacological lines of evidence indicate that cholinergic dysfunction plays an important role in age-related memory disturbances in humans and in animals. A number of neurotransmitter and neuropeptide systems in both cortical and subcortical brain regions are compromised in Alzheimer-type dementia (Cacabelos, 1996; Maelicke & Albuquerque, 1996; Panula *et al.*, 1995; Roberts & Lazareno, 1989). In most cases of Alzheimer's disease, the cholinergic innervation of the cerebral cortex is degenerated, and in some cases, the noradrenergic and serotonergic innervations are also reduced (Meguro *et al.*, 1995). The H_3 antagonist thioperamide, and the histamine precursor histidine, could be of therapeutic importance in this pathophysiological condition since they both have been shown to have ameliorating effects on learning and memory deficits induced by scopolamine (Miyazaki *et al.*, 1995; Miyazaki *et al.*, 1995).

A recent upregulation of the H_3 receptor in the hippocampus of aged rats was observed (Alves-Rodrigues *et al.*, in preparation). In the same study, no differences were observed in the number of receptors present in the striatum, and a small downregulation was seen in the cortex. These findings support the hypothesis of possible involvement of H_3 receptors in (patho)physiological conditions associated with hippocampal cognitive functions. These new observations would explain the beneficial effect of thioperamide as a memory and learning enhancer under pathological conditions in rodents where cholinergic transmission is deficient (Miyazaki *et al.*, 1995; Miyazaki *et al.*, 1995), while under normal cholinergic transmission thioperamide does not seem to significantly affect cognitive functions (Miyazaki *et al.*, 1995).

3.4.5 Regulation of glutamate NMDA-currents. Implications in cognitive functions.

Because the "laying down" of memories may be an important component of attentiveness and because the hippocampus participates in the formation of memory, the question arises as to whether histamine might affect hippocampal function. It has been shown that histamine reduces a calcium-dependent K^+ conductance in pyramidal neurons in areas CA_1 and CA_3 (Haas & Konnerth, 1983), (see figure 1.2.C). Bekkers reported additional kinds of neuromodulation by histamine in the hippocampus. Not only did histamine increase the amplitude of the NMDA autaptic current, it also accelerated its rate of decay (Bekkers, 1993; Haas *et al.*, 1995). The histamine-enhanced current was blocked by D-APV, indicating that the enhanced current was probably regulated by the

glutamate NMDA channels. The histamine enhancement was rapidly reversible. H_1 and H_2 receptors were not involved because the histamine enhancement was unaffected by the H_1 antagonists mepyramine and promethazine or by the H_2 antagonist cimetidine. Furthermore, the H_1 agonist 2-thiazolylethyl-amine and the H_2 agonists impromidine and dimaprit produced no enhancement of the autaptic current. On the other hand, the H_3 antagonist (and H_2 agonist) impromidine blocked the histamine enhancement but also reduced the size of the NMDA current to about half. In addition, the H_3 agonist (R)-α-methylhistamine mimicked the enhancing effect of histamine. These results suggest that H_3 receptors might mediate this effect. Surprisingly, however, the more selective H_3-antagonist, thioperamide, was unable to block the histamine enhancement of NMDA-currents in the same cells in which impromidine was effective (Bekkers, 1993). Moreover, it was found that enhanced currents due to exogenous histamine, differed qualitively from that due to increased release of neurotransmitter, which may mean that histamine does not modulate the release process.

It is also possible that histamine acts by altering the binding of glycine to its site on the NMDA receptor, althought Bekkers (1993) suggests that histamine enhancement of NMDA-currents and glycine modulation act by separate mechanisms. Histamine current enhancement probably resides mainly in a postsynaptic modification of NMDA channels.

Bekkers' results show that histamine potently modulates the gating of NMDA channels, increasing the amplitude and rate of decay of the NMDA component of synaptic transmission. In view of the lack of blockade by the H_3 antagonist thioperamide, a binding site different from the known histamine receptors seems to be involved (Bekkers, 1993; Bekkers *et al.*, 1996). Other amines have been shown to exert a similar potentiating effect on NMDA currents. In the light of the known affinity of histamine to a polyamine recognition site this site could be the locus of action, as well.

By selectively enhancing the NMDA component of neurotransmission, histamine should enhance processes in which NMDA currents participate, such as the triggering of long-term potentiation (LTP) (see Bekkers, 1993 for further references). Conversely, pathological conditions that deplete histamine in the brain might lead to a reduced ability to trigger LTP and, thus, to memory loss. There is increasing evidence for the involvemnt of NMDA receptors in other forms of computation in the central nervous system. It is therefore conceivable that histamine participates in such high-level functions as the control of arousal and attentiveness through its action on the NMDA channel (Bekkers, 1993). These features could have significant clinical interest in diseases such as attention deficit disorder (ADD) and sleep disorders.

Enhancing NMDA currents in the hippocampus through an increase in histamine release by administration of H_3 receptor antagonists or blocking the H_3 mediated inhibition of acetylcholine release by the same compounds could have important effects in

improving cognitive performances particularly in conditions where cognitive impairments occur such as ageing or Alzheimer's disease.

3.4.6 Regulation of the release of other neuromodulators

Besides the regulation of the release of the monoaminergic neurotransmitters, there is also a number of peptide transmitters in which the histaminergic system plays a modulatory role.

Enkephalin-YGG

In the striatum, a region containing abundant enkephalinergic neurons and histaminergic nerve terminals, (R)-α-methylhistamine and thioperamide exerted opposite effects on the levels of the tripeptide Tyr-Gly-Gly (YGG). YGG represents a characteristic extracellular metabolite of enkephalin (Llorens-Cortes *et al.*, 1986) and a reliable index of enkephalin release. The increase in YGG levels elicited by (R)-α-methylhistamine was not mimicked by H_1 and H_2 receptor antagonists, rendering unlikely an indirect effect due to a reduction of histamine level at the vicinity of enkephalinergic neurons (Schwartz *et al.*, 1991). Supporting this hypothesis was the lack of reversion of the effect of thioperamide by the brain penetrating H_2 receptor antagonist zolantidine. In fact, zolantidine alone elicited also a decrease in YGG levels, suggesting that H_2 receptors together with H_3 receptors might participate in the modulation of enkephalinergic neurotransmission. In contrast to the inhibitory effects of histamine previously described to be mediated by H_3 heteroreceptors, these H_3 receptors mediate a stimulatory effect on enkephalinergic neurons and this effect seems exerted under physiological conditions (Schwartz *et al.*, 1991). These findings may indicate the involvement of histamine on the enkephalinergic-induced feeling of pleasure and satiety.

Somatostatin

A recent report published by Puebla & Arilla (1996), showed that the histamine H_3 receptor antagonist thioperamide increases the number of somatostatin (SS) receptors in the frontoparietal cortical membranes and the content of somatostatin like immunoreativity. These effects were prevented by the H_3 agonist (R)-α-methylhistamine. The exact physiological significance of this observations cannot be stated at this time. However, as both histamine and and SS exert similar effects on cognitive processes, arousal behaviour, locomotor activity, and seizure susceptibility (see Puebla & Arilla (1996), for references), these findings indicate that neuronal histamine may (partly) modulate the above mentioned behaviours through interaction with the somatostatinergic system in the prefrontal cortex, a brain region rich in both SS (Puebla & Arilla, 1996) and H_3 receptors (see section 3.2).

3.5 Compounds with therapeutic potential in the CNS

From a therapeutic point of view, both H_3 agonists and antagonists are interesting. Besides acting on the histamine H_3 autoreceptor and thus modulating the histaminergic activity, these agents can also interact with histamine H_3 heteroreceptors, which will lead to the modulation of the release of other neurotransmitters. From the neurophysiological point of view, potential therapeutically relevant H_3-ligands fall into two categories. H_3-agonists: those compounds which will decrease synthesis and release of histamine and will decrease the overall histaminergic activity when acting on histamine H_3 autoreceptors, and, concomitantly may decrease the release of other neurotransmitters *via* histamine H_3 heteroreceptors; and H_3-antagonists: compounds which will increase the histaminergic activity when acting on the histamine H_3 autoreceptors, and may increase release of other neurotransmitters *via* histamine H_3 heteroreceptors.

In 1991, Arrang and collaborators reported on preliminary clinical studies performed using the H_3-agonist (R)-α-methylhistamine. In human volunteers receiving 175 mg (R)-α-methylhistamine p.o., the plasma levels of the drug reached a maximum at about 2 h and decayed with an apparent half-life of 1 hour; up to 6-8 hours. Drug plasma levels were high enough to be consistent with a persisting stimulation of H_3 receptors (Arrang *et al.*, 1991). Phase I clinical studies showed (R)-α-methylhistamine to be extremely well tolerated at dosages of several hundred milligrams per day during several days. In the CNS, the slow-wave sleep promoting activity of (R)-α-methylhistamine in cats (Lin *et al.*, 1990) was not observed in rodents (Monti, 1993). In humans, the drug did not elicit hypnotic activity even at high dosage reducing the interest in H_3- agonists as potential sleep enhancers.

In view of the latest findings involving the H_3 receptor with cognitive functions (see sections 3.3.1 and 3.4.4., for detailed information), H_3-antagonists are the most promising compounds concerning potential therapeutics. Unfortunately, the standard H_3-antagonist thioperamide and the newer H_3 -antagonist clobenpropit (which shows a 10 fold higher *in vitro* affinity for the H_3 receptor than thioperamide) are a thiourea-containing imidazoles. Earlier thiourea-containing drugs (e.g., H_2 antagonists) were found to be associated with unacceptable side effects (Brimblecombe *et al.*, 1973; Forrest *et al.*, 1975). Introduction of these ligands for clinical use is, therefore, unlikely. In contrast, development of non-thiourea H_2 histaminergic agents provide safe effective therapies (e.g., cimetidine), and non-thiourea-containing H_3 antagonists may, therefore, be good clinical candidates. Tedford *et al.* (1995) recently developed a new histamine H_3 antagonist, GT-2016, in which the thiourea moiety has been replaced by an amide group (see figure 3.2.). GT-2016 was shown to maintain a high degree of selectivity for the histamine H_3 receptor vs. the H_1 and H_2 subtypes. Furthermore, GT-2016 was absorbed orally and subsequently distributed to the brain. Receptor profiles revealed no appreciable

affinity for a wide variety of neurotransmitter, neuropeptide or second-messenger systems. Cross reactivity was not seen with GT-2016 for either histamine or *t*-methylhistamine. Using a microdyalisis study it was possible to measure increasing levels of histamine after GT-2016 in the rat cerebral cortex. Although this compound has entered phase I of clinical tests, future research has to confirm the advantages of this compound over the 'classical' H_3 antagonist thioperamide.

Figure 3.2. Structure of GT-2016 and thioperamide (Tedford *et al.*, 1995).

Within the last year several new H_3-antagonists have been introduced in the literature. Schlicker and colleagues (1996) studied the potencies of antagonists chemically related to iodoproxyfan. Curiously, in view of the previous study published by Ligneau *et al.* (1994) showing only antagonistic properties for iodoproxyfan, Schlicker and co-workers observed partial agonistic activities for this compound both in the mouse brain and the guinea-pig ileum. In their study, this group also showed that although none of the newly synthesised iodoproxyfan analogues had a higher affinity at the H_3 receptor than iodoproxyfan, these ligands, particularly the ethers, are H_3 antagonists of high or moderate affinity. The therapeutic potential of these compounds remains unstudied. Furthermore, Leurs *et al.* (1996), recently reported on impentamine (Leurs *et al.*, 1996). This compound is one of the highly potent H_3-antagonists which appears to discriminate between central and peripheral H_3-receptors. Structurally, impentamine is a histamine homologue that does not possess the (iso)thiourea moiety of thioperamide and clobenpropit, which makes it an attractive compound for future therapeutic development.

4. Concluding remarks

Neuronal histamine is involved in several physiological conditions, such as arousal, locomotor activity, food intake, and learning and memory processes. Thioperamide, an H_3 receptor antagonist, enhances neuronal histamine release and, therefore, activates, via stimulation of postsynaptic histamine H_1 and H_2 receptors, the histaminergic neuronal system. The opposite effects are accomplished by administration of histamine H_3

agonists, such as (R)-α-methylhistamine. Equally interesting was the discovery of histamine H_3 heteroreceptors that play a similar regulatory role as the autoreceptors, but now on the release of other neurotransmitters. Histamine H_3 heteroreceptors have been identified on various monoaminergic, nerve terminals, such as the noradrenergic, dopaminergic, serotonergic, and cholinergic.

Adjustment of noradrenaline release, via histamine H_3 heteroreceptors, can lead to therapeutic benefits. H_3 receptor agonists, decreasing the noradrenaline release, could be clinically interesting in the treatment of psychotic disorders, or as sleep enhancers, whereas histamine H_3 antagonists enhancing noradrenaline release could be useful as antidepressant agents. The existance of such a central noradrenaline release modulated by H_3-receptor has been observed in humans.

The dopaminergic neuronal system is known to be involved in two major pathologies. In schizophrenia, the dopaminergic system is found to be hyperactive and can be directly attenuated with dopamine antagonists, or indirectly using histamine H_3 receptor agonists *via* inhibition of dopamine release. Yet, clozapine, a drug unique in its efficiency on the treatment of neuroleptic resistant schizophrenia, shows a complex pharmacological profile binding with moderate to high affinity to several monoaminergic receptors (including antagonism at the histamine H_1 and H_3 receptors). This is one more example, among numerous others, of a complex multi-transmitter and multi-*loci* process underlying the development and onset of brain disorders where monoaminergic systems are known to be involved. In Parkinson's disease, on the other hand, the dopaminergic neuronal system in the *substantia nigra* is degenerated, and dopamine levels in the brain are abnormaly low; administration of H_3-antagonists, to increase dopamine release, could be helpful in attenuating Parkinsonian symptoms.

Modulating serotonin release could also have some therapeutic approaches. Since most succesful antidepressants are serotonin reuptake inhibitors (e.g. fluoxamine), it is suggested that histamine H_3 antagonists could potentially play a similar role in the treatment of depression by enhancing serotonin release.

Presumably the most important clinical aspect related to H_3-receptors, is its involvement in cognitive functions. Histamine was found to have a stimulating effect on the NMDA receptor. By enhancing the NMDA component of neurotransmission, histamine should enhance processes in which NMDA currents participate, such as the triggering of long-term potentiation (LTP). Conversely, pathological conditions that deplete histamine in the brain might lead to a reduced ability to trigger LTP and, thus, to memory loss. Moreover, acetylcholine levels in the hippocampal formation can be increased by H_3-antagonists, which is of particular interest for improvement of memory loss processes that occur under pathological conditions in which a defect cholinergic neuronal system is present.

Sofar, no H_3-ligand has yet been introduced in therapeutics, nevertheless, the development of the non-thiourea, brain penetrating H_3 antagonists such as GT-2016, is a promising step towards clinical appliance of H_3 ligands.

5. Scope of this thesis

As can be understood from what has been described in this introductory chapter, the histamine H_3 receptor is an important modulatory centre of general brain activity. Its actions include regulation of the histaminergic neuronal system (in which it acts as an autoreceptor) as well as several other neuronal systems such as noradrenergic, serotonergic, cholinergic, and dopaminergic (Arrang *et al.*, 1983; Schlicker *et al.*, 1994). The histamine H_3 receptor has not been cloned, yet. Therefore, studies on this receptor can only be based on the pharmacological data obtained from ligands that bind to the H_3-receptor with high affinity and selectivity. Fortunately, numerous of these ligands have been synthesised and made available over the last decade (Leurs *et al.*, 1996; Leurs *et al.*, 1995; Schlicker *et al.*, 1996). In view of several recent reports published on cognitive enhancing properties of H_3 receptor antagonists, interest on H_3 ligands as potential therapeutical agents has dramatically increased.

It has been shown that histaminergic activity is altered in several brain disorders such as Schizophrenia (Prell *et al.*, 1995) and Alzheimer's disease (Cacabelos *et al.*, 1989; Fernandeznovoa *et al.*, 1994; Mazurkiewicz-Kwilecki & Nsonwah, 1989; Panula *et al.*, 1995). Nevertheless, the SAR of neuroleptics used in therapeutics of those pathologies have not been characterised for the H_3 receptor. In our study we used several typical and atypical neuroleptics and assessed them for their affinity and functionality on the H_3 receptor (Chapter 2). In view of a high to moderate affinity observed for clozapine, and in order to understand SAR requirements for the binding of non-imidazole compounds to the H_3 receptor, we studied the structure affinity requirements of a series of 20 clozapine analogues (including the two major clozapine metabolites) for binding to the H_3 receptor (Chapter 2).

Following up investigations on the possible existence of H_3 receptor subtypes (Leurs *et al.*, 1996; Clapham & Kilpatrick, 1992; Schworer *et al.*, 1994; West *et al.*, 1990) and considering the therapeutical interest of developing H_3-receptor ligands with discriminative properties between H_3 receptor subtypes, we (in Chapter 3) characterised the binding and functional properties of two homologues of the potent H_3-receptor agonist immepip in two different binding assays and two distinct functional systems.

Albeit thioperamide was the first ligand shown to have high affinity and high selectivity for the H_3 receptor (Arrang *et al.*, 1987) and is still used as the classical H_3-

receptor antagonist, in this study the radiolabelling synthesis and the characterisation of [^{3}H]-thioperamide as a radioligand is firstly described (Chapter 4).

In view of the recent reports involving H3 receptor antagonism with enhancement of hippocampal memory formation (De Almeida & Izquierdo, 1986; Meguro *et al.*, 1995; Miyazaki *et al.*, 1995; Miyazaki *et al.*, 1995), we pharmacologically characterised the histamine H3 receptor in the rat hippocampus using receptor binding studies and the H3-receptor modulation of noradrenaline release (Chapter 5). Once established the pharmacological characteristics of the hippocampal H3 receptor, we investigated, in Chapter 6, age-related changes in the density and functionality of the histamine H3 receptor in several areas of the rat brain. Histamine levels were measured in nine brain regions of both young and old rats. The (patho)physiological implications of age-related changes on the histamine H3 receptor were discussed in Chapter 6.

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Chapter 2

INTERACTION OF CLOZAPINE AND OTHER NEUROLEPTICS WITH THE HISTAMINE H3 RECEPTOR. STRUCTURE AFFINITY RELATIONSHIPS FOR THE BINDING OF CLOZAPINE METABOLITES AND ANALOGUES TO THE HISTAMINE H3 RECEPTOR

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Summary

There is evidence that histaminergic activity in the brain is altered in several mental disorders including schizophrenia (Deutsch *et al.*, 1993; Kaminsky *et al.*, 1990; Martinez-Mir *et al.*, 1993; Prell *et al.*, 1995). We examined possible interactions between several neuroleptics, such as the atypical agent clozapine, and the histamine H3 receptor. In competition binding assays using the H3 antagonist [^{125}I]-iodophenpropit, we observed a moderate to high affinity of clozapine for the H3 receptor. The dissociation constant obtained for clozapine was 236 ± 87 nM. Functionally, clozapine acted as an antagonist against the inhibition of electrically stimulated release of [^{3}H]-5-HT mediated by histamine H3 receptors. The K_B value obtained for clozapine was 80 nM.

We also tested the affinity of several clozapine metabolites and analogues for the histamine H3 receptor. Qualitative structure affinity relationships were derived for the tested compounds. In the clozapine molecule structurally different moieties were considered. In comparision with the affinity for the H3 receptor shown by clozapine, the following main conclusions can be drawn: the 4-piperazinyl region does not allow substituents longer than a CH_3 or electronegative atoms such as an O; substitutions at the 5-diazepine position do not drastically alter the affinity for the H3 receptor, although a basic nitrogen is favoured over CH_2, O, or S; the 8 position in phenyl ring I is an important modulatory site for H3 affinity, electronegative substituents such as chloro and fluoro in this aromatic group increase the affinity. When these substituents are present at the phenyl group II, these substituents totally disable binding to the H3 receptor.

If the K_i value for clozapine at the H3 receptor is similar in rat and man, than brain clozapine concentrations might be high enough for a functional interaction with the H3 receptor under clinical conditions. The two major clozapine metabolites (clozapine-N-oxide, and N-desmethylclozapine) will not be responsible for a possible contribution of the H3 receptor antagonism to the clinical profile of clozapine.

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Introduction

The histamine H_3 receptor was first described by Arrang *et al.* (1983) as a presynaptic regulatory system for the release of histamine in rat brain. In recent years it was found that H_3 receptors are also present on nerve terminals of other monoaminergic neurons (such as serotonine, noradrenaline, and dopamine) where it acts as an heteroreceptor modulating the release of these neurotransmitters (for review see Schlicker *et al.*, 1994). These findings have opened new perspectives in the design of selective H_3 compounds as potential drugs (Leurs & Timmermann, 1992; Leurs *et al.*, 1995; Schwartz *et al.*, 1990).

Clozapine is a so called atypical antipsychotic agent of the tricyclic dibenzodiazepine class, whose clinical efficacy in the treatment of drug resistant schizophrenia is thought to be associated with both dopaminergic and serotonergic systems (O'Dell *et al.*, 1990). Compared with classical neuroleptics, the affinity of clozapine for the dopamine D_2 receptor is low (Brucke *et al.*, 1992; Karbe *et al.*, 1991). On the other hand, clozapine's affinity for dopamine D_1 and D_4 receptors is relatively high, and it binds preferentially to mesolimbic rather than neostriatal dopaminergic neurons (Farde *et al.*, 1994; Pehek & Yamamoto, 1994; Van Tol *et al.*, 1991). Clozapine also has high affinity for the histamine H_1 receptor, the α_1- adrenergic receptor, and a moderate affinity for the α_2- and β-adrenergic receptors (Baldessarini *et al.*, 1992).

Recent studies suggest that the neurotransmitter histamine may be involved in schizophrenia (Kaminsky *et al.*, 1990; Deutsch *et al.*, 1993; Prell *et al.*, 1995). In treatment resistent schizophrenic patients, the mean level of tele-methylhistamine (*t*-MH) in cerebrospinal fluid, an index of histaminergic activity, was 2.6 fold higher than in controls (Prell *et al.*, 1995). Among schizophrenic patients, cerebrospinal fluid levels of *t*-MH correlated positively with those of other neurotransmitters metabolites, and correlated positively with severity of schizophrenic symptoms (Prell *et al.*, 1995). Consonant with increased histaminergic activity is a reduction in the density of H_1 receptors in postmortem frontal cortex of schizophrenic patients (Nakai *et al*, 1991) and the reported therapeutic actions of famotidine, an H_2 antagonist in schizophrenic inpatients (Kaminsky *et al.*, 1990; Deutsch *et al*, 1993). Evidence for elevated histaminergic activity suggests possible functional anomalies in H_3 receptors. Therefore, we examined the affinity of clozapine, and several other neuroleptics, for the H_3 receptor.

Methods

Preparation of membranes from rat cerebral cortex

Male Wistar rats (200-220 g) were decapitated and the brains were rapidly removed. The cortices were dissected and homogenised in 10 volumes (v:w) of ice-cold 50 mM Tris-HCl buffer (with 5 mM $MgCl_2$, 145 mM NaCl, pH 7.4 at 4°C) using a Ultra-Turrax blender and a Potter-Elvhjem homogeneizer. This homogenate was centrifuged at 800 g for 10 min. The pellet was discarded and the supernatant centrifuged at 40,000 g for 40 min. The resulting pellet was rinsed twice under the same conditions. The final pellet was resuspended in 1.5 volumes of the Tris-HCl buffer and stored at -80°C until the day of the experiment, when it was diluted 5 times (v:w) in the same solution (pH 7.4, 37°C).

Competition binding assays

Binding assays were based on the procedure described by Jansen *et al.* (1994). The experiments were performed in a final volume of 500 µl using polyethylene tubes. The incubation at (37°C, 60 min) was started by addition of 100 µl of membrane suspension (40-120 µg) and stopped by rapid filtration through Whatman GF/C filters pretreated with 0.3 % polyethyleneimine using a Brandel harvester. The filters were washed twice with 3 ml of ice-cold Tris-HCl buffer (with 5 mM $MgCl_2$, 145 mM NaCl, pH 7.4 at 4°C). Competition experiments were done using 0.25 nM [^{125}I]-iodophenpropit (specific activity 1950 Ci/mmol). The radioactivity bound to the filters was measured in a LKB gamma counter.

Non-specific binding was defined with 0.3 µM thioperamide as in Jansen *et al.* (1994). K_i values were calculated from the respective IC_{50} values by applying the Cheng-Prusoff (1973) equation: $K_i=IC_{50}/((1+[L])/Kd)$ (Cheng & Prusoff, 1973) where K_i represents the dissociation constant of the unlabeled ligand calculated from competition binding experiments, IC_{50} is the concentration of unlabeld ligand that displaces 50% of the specific binding, [L] is the concentration (in mol/liter) of the labelled radioligand used in the competition experiments, and K_d the dissociation constant of the labelled ligand as determined from saturation binding assays.

Competition binding experiments were evaluated on a Macintosh computer using the non-linear curve fitting programme LIGAND (Munson & Rodbard, 1980). With the aid of this programme binding curves were, respectively, fitted (unweighted) to a one and two independent sites models. The improvement of the fit for the model with additional parameters was evaluated based on the 'extra sum of squares' principal (Draper & Smith, 1966) taking $p<0.05$ to be significant.

Functional assays

Based on Van der Werf *et al.* (1987), neocortex slices (0.3mm x 0.3mm) from male Wistar rats were incubated for 15 min in 2 ml Krebs-Ringer buffer (in mM: NaCl 118, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.4, $NaHCO_3$ 25, glucose 10, pH 7.4 at 37° C when gassed with O_2/CO_2 (95%/5%)), containing 5 µCi [^{3}H]-5-hydroxytryptamine (11Ci/mmol). After washing 4 times with 10 ml KRB, the slices were perfused for 60 min at 0.3 ml/min under constant gassing (95% O_2, 5% CO_2). Subsequentely, five 10 min fractions were collected, tritium overflow was evoked by electrical stimulation (rectangular pulses of 30 mA and 2ms, 1Hz) during the whole second fraction. The antagonists and the agonist were added one hour and 30 minutes before stimulation, respectively. Finally, the slices were perfused for 10 min with 0.1N HCl to extract the remaining content of tritium in the tissue. 3ml liquid scintillation cocktail was added to each tube. Radioactivity was counted in a Packard beta counter.

EC_{50} values were determined according to the equation $E=100-(100-E_{max})/(1+(EC_{50}/[A])$ where E represents the effect observed at any agonist concentration of [A], EC_{50} represents the agonist concentration that produces half-maximal effect, and E_{max} the maximal effect. The dissociation constant (K_B) was calculated from the equation $[A']/[A]=1+[B]/K_B$, where [A] is the concentration of the agonist that produces half maximal effect, [A'] is the concentration of the agonist that produces half maximal effect in the presence of the antagonist concentration [B].

Chemicals

[^{125}I]-iodophenpropit was labelled to a specific activity of 1950 Ci/mmol as described by Menge *et al.*, (1992). [^{3}H]-5-hydroxytryptamine (11Ci/mmol) was purchased from Amersham. Thioperamide maleate was synthesised at the Department of Pharmacochemistry (Vrije Universiteit, Amsterdam), polyethyleneimine was purchased from Aldrich-Chemie, compounds **6**, **9**, **17**, and **18**, were purchased from RBI. Compounds **15** and **16** were generously provided by Prof. Dr. Hakan Wikström (RU, Groningen). All other clozapine analogues were kindly provided by Sandoz, Netherlands. The other neuroleptics tested were purchased from RBI.

Results

According to results previously reported by our research group (Jansen *et al.*, 1994), the dissociation constant of [^{125}I]-iodophenpropit for the histamine H_3 receptor in rat brain cortex is 0.57 nM. In competition binding experiments described in this report, 0.25 nM [^{125}I]-iodophenpropit was used to label the histamine H_3 receptor. Non-specific binding represented 50-60% of total binding at this concentration of [^{125}I]-

iodophenpropit. Displacement of 0.25 nM [^{125}I]-iodophenpropit binding by clozapine (Figure 1A) fitted best to a one site model (P<0.05) using the non-linear curve fitting program LIGAND. The K_i value obtained for clozapine was 236 ± 87 nM (n=6, mean ± s.e.mean). The K_i values of other neuroleptics tested for their affinity for the H_3 receptor are shown on Table 1.

Table 1 Affinity of H_3 receptor agonists and antagonists and of several neuroleptics for [^{125}I]-iodophenpropit binding sites to rat cortex compared with their functional potency (apparent values of K_B and K_i are shown assuming competitive antagonism). For the binding studies data shown are mean±s.e.mean. The values between parenthesis represent the number of independent experiments. Functional data represent the value obtained from the fitting of mean data (3-4 experiments).

Ligand	*Inhibition of [^{3}H]-5-HT release*	*[^{125}I-iodophenpropit binding*
Histamine	EC_{50}=48 nM (3)	K_{iH}=38±10 nM (4)a)
(R)–α–methylhistamine	EC_{50}=6.0 nM (4)	K_{iH}=3.5±1.2nM(4)a)
Thioperamide	K_B=1.1 nM (3)	K_i=4.3±1.6 nM (7)a)
Impromidine	K_B=20 nM (3)	K_i=51±9 nM (3)a)
Clozapine	K_B=80 nM (4)	K_i=236±87 (6)
Chlorpromazine	N.D.	K_i>20µM (2)
Haloperidol	N.D.	K_i>20µM (2)
Spiperone	N.D.	K_i>20µM (2)
Seroquel	N.D.	Ki>20µM (3)
Olanzapine	N.D.	Ki>20µM (3)

N.D.: Not determined

a) Jansen *et al*, 1994

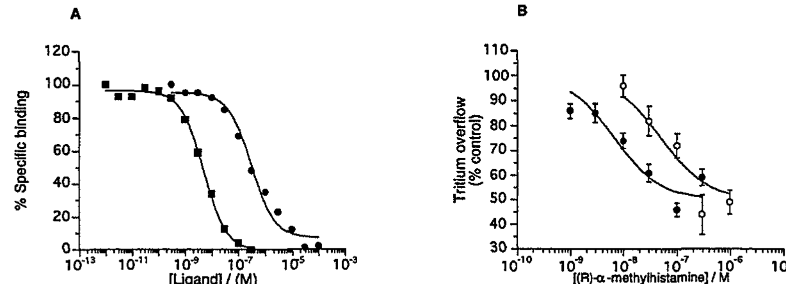


Figure 1 (A) Inhibition of $[^{125}I]$-iodophenpropit binding (0.25 nM) by the H_3 receptor antagonist thioperamide (filled squares) and clozapine (filled circles). Data are expressed as % specific binding determined with 0.3 μM thioperamide, which represented 50%-60% of the total binding.The curves show one site fittings of a single representative experiment (n=6) with triplicate determinations. (**B**) Inhibition of the electrical stimulated release of $[^{3}H]$-5-HT from rat brain neocortex slices by (R)–α–methylhistamine in the absence (filled circles) and in the presence of 1 μM clozapine (open circles). Each point represents mean ± SEM of three or four different experiments performed in duplicate.

Qualitative structure affinity relationships of clozapine analogues

Following the observation that clozapine interacts with the H_3 receptor, the affinities of twenty clozapine analogues for the histamine H_3 receptor were determined (Table 2). Two of these compounds, clozapine-N-oxide (compound **19**, Table 2), and N-desmethylclozapine (compound **6**, Table 2) represent two major metabolites that are formed *in vivo* after clozapine administration (Figure 2). Modifications on the clozapine molecule are seen in five regions (see structure on Table 2): 4-piperazinyl region (X_1), 5-diazepine region (X_2), 8-phenyl region (X_3), 2-phenyl (X_4) and 10-diazepine (X_5).

When clozapine is demethylated at position X_1, one of the two possible pathways of the metabolism of this drug in humans (Wagstaff & Bryson, 1995), the resulting N-desmethylclozapine (compound **6**, Figure 2) displayed an affinity 50 fold lower (K_i= 10±2.4 μM, Figure 3A) than clozapine. For compound **19**, Clozapine N-oxide, the second major metabolite of clozapine (Figure 2) the affinity for H_3 is almost completed lost after oxidation at position X_1 (Figure 3A). The introduction of an hydroxyl group in the alkyl substituent in position X_1 decreased the affinity, although a propanol group (**4**, K_i=6.7±0.01 μM) seems to be better accepted in this position than an ethanol substituent (**3**, K_i>20 μM). Compound **5**, clozapine methylated at position X_2, showed a considerable affinity for the histamine H_3 receptor (K_i= 0.4±0.1 μM), comparable with the value observed for clozapine (K_i= 0.2±0.1 μM). In compound **1** (K_i=7.7±3.0 μM) and **2** (K_i=10±4.1 μM) electronegative groups (oxygen and sulphur, respectively) are present in position X_2 instead of a basic amine as in clozapine.

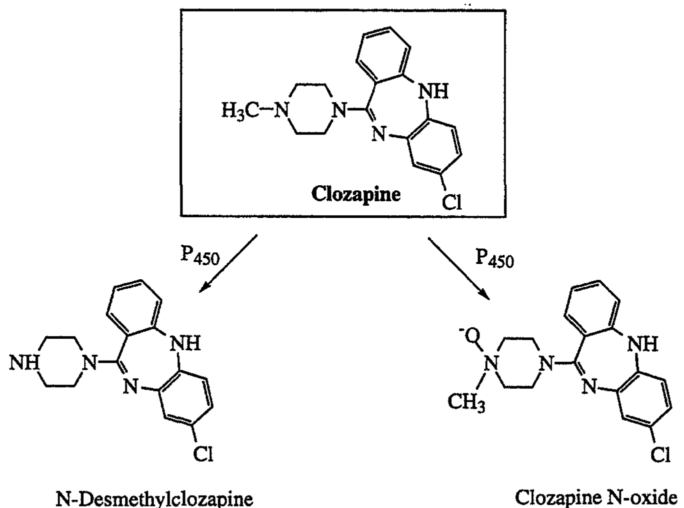
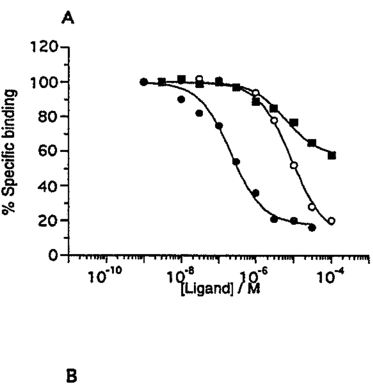


Figure 2 The main metabolites (human liver) of clozapine (Wagstaff & Bryson, 1995).



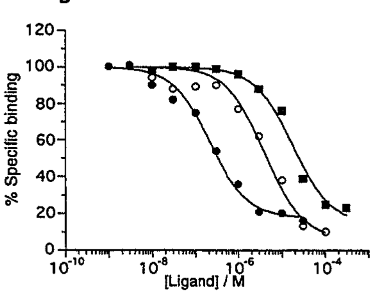


Figure 3 Inhibition of [^{125}I]-iodophenpropit binding (0.25 nM) by (A) clozapine (filled circles); and clozapine metabolites: N-desmethylclozapine (open circles), and clozapine-N-oxide (filled squares). (B) clozapine (filled circles), compound **1** (open circles), compound **9** (filled squares). The curves show one site fittings of a single representative experiment (n≥3) in triplicate.

Table 1 Affinity of clozapine analogues for the histamine H_3 receptor.

comp. no	X_1	X_2	X_3	X_4	X_5	K_i (μM)
clozapine	CH_3	NH	Cl	H	-N	0.2±0.1
1	CH_3	O	Cl	H	-N	7.7±3.0
2	CH_3	S	Cl	H	-N	10.0 ± 4.1
3	$(CH_2)_2OH$	NH	Cl	H	-N	>20
4	$(CH_2)_3OH$	NH	Cl	H	-N	6.7 ± 0.0
5	CH_3	NCH_3	Cl	H	-N	0.4±0.1
6	H	NH	Cl	H	-N	10.0 ± 2.4
7	CH_3	CH_2	F	H	-N	5.7±1.1
8	CH_3	S	H	Cl	-N	>20
9	CH_3	O	H	Cl	-N	>20
10	H	CH_2	H	H	-CH	>20
11	CH_3	CH_2	H	H	-N	>20
12	CH_3	NH	H	H	-N	>20
13	CH_3	NH	CH_3	H	-N	>20
14	CH_3	NH	OSO_2CF_3	H	-N	>20
15	CH_3	NH	H	OSO_2CF_3	-N	>20
16	CH_3	O	H	OSO_2CF_3	-N	>20
17	CH_3	S	H	Cl	-N	>20
18	H	O	H	Cl	-CH	>20
19	CH_3, N-oxide	NH	Cl	H	-N	>20
20	$(CH_2)_2O(CH_2)_2OH$	S	H	H	-N	>20

(* mean±SEM, n≥3 independent experiments)

These derivatives show an approximately 50 fold decrease in the H_3 receptor affinity in comparision with clozapine. From compounds **12**, **13**, and **14** we conclude that substituting the chloro at position X_3 for an hydrogen (**12**), a methyl group (**13**), or a OSO_2CF_3 (**14**) result in a profound decrease on the affinity for the H_3 receptor (all K_i values above 20 μM). A similar finding is observed from comparison of the affinities of compounds **1** (K_i=7.7±3.0 μM) and **9** (K_i>20 μM). Moving the chloro atom from position X_3 to position X_4 significantly reduced the affinity of compound **9** (Figure 3B)

relatively to compound **1** (Figure 3B). The same is observed for compounds **2** (K_i=10±4.1 μM) and **8** (K_i>20 μM). In position X_4, electronegative groups such as a chloro atom or OSO_2CF_3 are not favourable for binding to the histamine H_3 receptor.

Functional studies

The electrically evoked tritium overflow was inhibited in a concentration dependent manner by histamine and the H_3 agonist (R)–α–methylhistamine (Table 1, Figure 1B). H_3-antagonists inhibited the (R)–α–methyhistamine effect with potencies comparable to their affinity for the [^{125}I]-iodophenpropit binding sites (Table 1). Clozapine (1 μM) shifted the (R)–α–methylhistamine concentration response curve to the right yielding an apparent K_B value of 80 nM. Clozapine (1 μM) by itself, did not affect the stimulated (104 ± 1%; n=3, mean ± SEM) or the basal release of [^{3}H]-5-HT (100±2 %; n=3, mean ± SEM.

Discussion

In the present work, we observed an intringuing interaction of the non-imidazole clozapine with the histamine H_3 receptor, as measured by radioligand binding and neurotransmitter release, where clozapine acts as an H_3 antagonist. Other neuroleptics tested in the binding assay had much lower affinities for the H_3 receptor. Similar results were reported by Kathmann *et al.* (1994).

Typical plasma concentrations of clozapine associated with clinical responses in man are approximately 0.6-1.2 μM (Baldessarini & Frankenburg, 1991). In rat, with doses higher than 5 mg/kg and within the therapeutic range in humans, clozapine levels in the brain averaged 29 times higher than the corresponding plasma drug level (Balderassini *et al*, 1993). Assuming total plasma levels of 0.9 μM and 95% protein bound, total brain levels of 1.3 μM can be obtained. If the K_i value for clozapine at the human H_3 receptor is similar to the value obtained for its rat counterpart, brain clozapine concentrations might be high enough for a functional interaction with the H_3 receptor under clinical conditions.

Probably due to the high affinity and selectivity demonstrated by several of the early introduced imidazole containing compounds such as thioperamide and (R)–α–methylhistamine for the H_3 receptor, the interest in non-imidazole compounds has been limited. Recently, with the discovery of the involvement of the histamine H_3 receptor in a variety of brain functions (Onodera *et al.*, 1994; Schwartz *et al.*, 1991), this receptor has become a target for development of selective ligands as potential therapeutic agents. As no structural information is available for this receptor yet, an approach based on comparison of the affinities/activities of structurally related compounds is the only way to

study ligand-binding characteristics. According to Leurs *et al.*, (1995) H_3 antagonists can be generally classified into four distinct groups: 1) elongated imetit and histamine analogues, 2) burimamide analogues, 3) thioperamide analogues, and 4) other ω-functionalized 4(5)-alkyl imidazoles. Lipophilicity is thought to play an important role in the binding of most antagonists to the H_3 receptor. Structural requirements for the binding of compounds which do not contain an imidazole to the H_3 receptor are not known. Therefore, we analysed the structural requirements for binding of various clozapine analogues and metabolites to the H_3 receptor.

Taking the available compounds, we considered distinct regions in the clozapine molecule for structural variations. From the data obtained, several conclusions can be drawn regarding affinity of analogues of clozapine for binding to the histamine H_3 receptor. None of the screened compounds showed higher affinity for the H_3 receptor than clozapine. In man, extensive clozapine metabolism occurs in the liver and yields several metabolites (mainly clozapine-N-oxide and N-desmethylclozapine, Figure 2), (Wagstaff & Bryson, 1995). After 4 weeks of treatment with a mean dosage of 350 mg/day, plasma ratios of N-desmethylclozapine (**6**) and clozapine N-oxide (**19**) concentrations over clozapine concentations are in the range of 0.6 to 1.0 and 0.2 to 0.3, respectively (Volpicelli *et al.*, 1993). Both these compounds displayed considerably lower affinity for the H_3 receptor than clozapine. Therefore we conclude that, if the H_3 receptor antagonistic properties of clozapine contribute to the clinical efficacy of this drug, the two major metabolites (N-desmethylclozapine and clozapine N-oxide) will not be responsible for such an effect. For H_3 receptor affinity the most favourable substituent in position X_1 is a methyl group and replacement by larger groups (compounds **3**, and **19**) drasticly reduces the affinity for the H_3 receptor. In position X_2, substituents such as NH or NCH_3 are preferred to more electronegative atoms such as S (**2**) or O (**1**). Compound **7** supports the observation that basic nitrogens are indeed the best substituents at position X_2. The affinity for the H_3 receptor seems to drop when a CH_2 is replacing the NH of clozapine in position X_2. This conclusion is, however, based on the fluoro analogue of clozapine and its CH_2 derivative. X_3 behaves as an important site of modulation of binding to the H_3 receptor. When other substituents than chloro or fluoro atoms are present in this position the H_3 receptor affinity disappears. This holds true for the replacement of the halogen by either a H (**12**), a OSO_2CF_3 (**14**) or a CH_3 (**13**). Electronic influences seem to be important at this position but when the group is too large (**14**) steric hindrance may occur and reduce the affinity for the H_3 receptor. The importance of the chlorine substituent may be attributed to its lipophilicity, which appears to play a relevant role in the binding of compounds to the H_3 receptor, regardless of the presence of imidazole structures. Similarly Van der Goot *et al.* (1992) noted that the introduction of a chloro substituent in the *para* position of a benzyl-substitutent of an imetit analogue (clobenpropit) increased the H_3 antagonistic activity by

more than 10 fold. It should be pointed out, though, that lipophilicity is not enough for binding to the H3 receptor. Position X4, e.g., seems to prefer an hydrogen over large electronegative groups such as chloro atoms, or OSO_2CF_3 groups. No conclusions could be drawn from position X5.

In summary, we report the affinity of clozapine, clozapine analogues, and other neuroleptics for binding to the histamine H3 receptor. Emphasis is placed on the observation that, due to the low affinity showed for the H3 receptor by the two major clozapine metabolites (N-desmethylclozapine and clozapine N-oxide), they will not account for the possible clinical relevance of the interaction of clozapine with the H3 receptor. The work presented in this chapter is also one of the first efforts to study structural requirements for H3 receptor binding by compounds that do not derive from histamine itself. For further understanding of the SAR of non-imidazole compounds for the H3 receptor, systematic design and testing of novel compounds in studies similar to the ones previously done for the numerous available imidazole containing H3 ligands (Leurs *et al.*, 1995; Lipp *et al.*, 1992) are required. In our study, we could only use compounds commercially available or generously provided to us by colleagues.

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Chapter 3

PHARMACOLOGICAL ANALYSIS OF IMMEPIP HOMOLOGUES. FURTHER EVIDENCE FOR H3 RECEPTOR HETEROGENEITY?

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Summary

Following a previous report by our research group (Leurs *et al.*, 1996) on discriminative properties of a series of aliphatic histamine homologues, we now studied immepip and its lower (VUF 4735) and higher (VUF 4929) homologues as ligands for the histamine H_3 receptor in two binding assays (using [^{125}I]-iodophenpropit and [^{3}H]-N^{α}-methylhistamine, as radiolabelled ligands) in rat cerebral cortex membranes, and two functional H_3 receptor models (inhibition of the neurogenic contraction of the guinea-pig jejunum and inhibition of [^{3}H]-noradrenaline release in rat cerebral cortex slices).

Immepip showed high affinity for the binding of [^{3}H]-NAMHA (pK_i=8.7) which was comparable to the high affinity component of the binding of [^{125}I]-iodophenpropit ($pK_{i(high)}$=8.5). VUF 4735 also showed similar affinities in both binding assays (pK_i=6.1). The binding data obtained for VUF 4929, however, showed major discrepancies between the two assays.

Functionaly, the immepip homologues acted as competitive H_3-receptor antagonists in both testing systems. The potencies (pA_2 values) observed at the guinea pig jejunum were 8.4 and 6.2 for VUF 4929 and VUF 4735, respectively, whereas on the electrically evoked release of [^{3}H]-noradrenaline from cortical slices the pA_2 values were 7.1 and 5.5 for VUF 4929 and VUF 4735, respectively. Moreover, the H_3-receptor agonist immepip, but not the H_3-agonist (R)–α–methylhistamine, showed almost a 10-fold higher agonistic potency in the rat cerebral cortex than in the guinea-pig jejunum. The differences in potencies shown by the ligands between the two functional assays are discussed in relation to receptor effector coupling and H_3 receptor heterogeneity.

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Introduction

It has been shown that the presynaptic H3 histamine receptor regulates the release of histamine and other monoamines and can be regarded as a potential target for new therapeutics (Leurs & Timmermann, 1992; Leurs *et al.*, 1995; Onodera *et al.*, 1994; Schlicker & Kathmann, 1994; Schwartz *et al.*, 1991). Results from several radioligand binding studies question the homogeneity of the H3 receptor binding sites. Based on the biphasic displacement of [^{3}H]-N$^{\alpha}$-methylhistamine binding by the H3 antagonists thioperamide and burimamide West *et al.* (1990) suggested $H3_A$- and $H3_B$-receptor subtypes, responsible for H3 receptor mediated inhibition of histamine release and the H3 receptor mediated inhibition of histamine synthesis, respectively (Arrang *et al.*, 1987; Arrang *et al.*, 1987). Yet, for some ligands the affinities for the putative $H3_A$- and $H3_B$-receptor subtypes do not explain the differences in functional potencies (Kathmann *et al.*, 1993; West *et al.*, 1990).

Also in studies with the recently developed radiolabelled antagonist [^{125}I]-iodophenpropit (Jansen *et al.*, 1992; Jansen *et al.*, 1994; Menge *et al.*, 1992) biphasic displacement, suggesting two receptor types, by some H3 receptor antagonists was found (burimamide and dimaprit), but, in contrast to the studies with [^{3}H]-N$^{\alpha}$-methylhistamine, for thioperamide monophasic displacement curves were obtained (Jansen *et al.*, 1992; Jansen *et al.*, 1994). These results question the subtype extension on the basis of [^{3}H]-N$^{\alpha}$-methylhistamine binding and also reinforce the complexity of receptor-ligand interactions.

Until recently, only a few potent and selective agonists for the histamine H3 receptor were described. Methylation of the side chain of histamine resulted in the most known and used H3 receptor agonist, (R)-α-methylhistamine, (Arrang *et al.*, 1983, 1987). More recently, new non-chiral, H3-agonists, imetit and immepip were described (Garbarg *et al.*, 1992; Howson *et al.*, 1992; Van der Goot *et al.*, 1992; Vollinga *et al.*, 1994). Immepip resulted from a series of histamine homologues in order to obtain information about the optimal location of the amino group relative to the imidazole ring (Vollinga *et al.*, 1995). In immepip, the alkyl side chain of histamine was extended to a length of four methylene groups, the amino group being incorporated in a piperidine ring (Table 1). Since then, and following up studies done with a series of histamine analogues (Leurs *et al.*, 1996; Vollinga *et al.*, 1994), two immepip analogues have been synthesised in which the alkyl chain between the imidazole ring and the piperidine ring varies (Vollinga *et al.*, 1995).

Recently, Leurs *et al.* (1996) published on the discriminative properties of the histamine homologues between two functional histamine H3 receptor assays. In their study, all histamine homologues studied acted as H3-antagonists in the guinea pig

jejunum. Intringingly, the propylene, butylene and pentylene homologues could discriminate between two functional H3-receptor models, the guinea pig jejunum and mouse brain, showing partial agonism in the brain. These data were discussed in relation to possible receptor heterogeneity. To further address the issue of H3 receptor heterogeneity, we performed a similar but more detailed pharmacological study on immepip and two of its homologues. To assess putative discriminative properties these compounds were examined in both [^{3}H]-N$^{\alpha}$-methylhistamine and [^{125}I]-iodophenpropit binding assays. Moreover, we compared the functional potencies of these compounds on the H3 receptor mediated inhibition of neurogenic contractions of the guinea-pig jejunum and on the H3 receptor mediated inhibition of electrically-stimulated release of [^{3}H]-noradrenaline in rat cerebral cortex slices.

Methods

H3 receptor binding studies

[^{125}I]-Iodophenpropit binding experiments were carried out essentially as described by Jansen *et al.*, (1994). Briefly, cerebral cortices obtained from male Wistar rats (200-220 gram) were homogenized in 15 volumes of ice-cold Tris-HCl buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 145 mM NaCl, pH = 7.4 at 4°C) using an Ultra-Turrax homogenizer (8 s) and a Potter-Elvehjem homogenizer (4 up and down strokes) and centrifuged at 1000g for 10 mins (4°C). The supernatant was centrifuged at 40,000g for 20 mins, the pellet was resuspended in the same buffer and the last centifugation step was repeated. The final pellet was resuspended in 1.5 volumes (v/w) of Tris-HCl buffer and frozen at -80°C. Before each experiment the membranes were resuspended in 7 volumes (v/w) of the incubation buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 145 mM NaCl, pH = 7.4 at 37°C).

The [^{125}I]-iodophenpropit binding assay was carried out in triplicate at 37°C in Tris-HCl buffer in a total volume of 500 µl, using polyethylene tubes. In the competition binding experiments 0.25 nM [^{125}I]-iodophenpropit was applied. Non-specific binding (40-50% of total binding) was determined in the presence of 0.3 µM thioperamide. In some experiments 10 µM of the GTP-analogue GTPγS was included in the test tubes. Incubations were started by the addition of 100 µl membranes (ca 30 µg of protein per tube) and were terminated after 60 min by rapid dilution with 2 ml ice-cold 50 mM Tris-HCl buffer (pH = 7.4 at 4°C) and filtration through polyethyleneimine (0.3%)-pretreated Whatman GF/C filters. The filters were subsequently washed twice with 2 ml of the same buffer. The radioactivity retained on the filters was determined by an LKB gamma counter.

$[^3H]$-N^{α}-Methylhistamine binding experiments were carried out essentially as described by Tedford *et al.* (1995). Briefly, cerebral cortices obtained from male Wistar rats (200-300 gram) were homogenised in 10 volumes (w/v) of ice-cold Krebs-Ringer HEPES buffer pH = 7.4 and centrifuged at 1000g for 10 min (4°C). The supernatant was centrifuged at 40 000g for 30 mins, the pellet was resuspended to give a final concentration of 2mg/ml. This suspension was stored at -80°C before use. The assay was performed in Na^+ phosphate buffer (50 mM, pH 7.4, 5 mM EDTA) in a final volume of 400 µl containing ca 150-200 µg of protein. For competition experiments $[^3H]$-N^{α}-Methylhistamine (80 Ci/mmol) was used at a concentration of 0.2 nM. The binding test (25°C) was initiated by the addition of 100 µl membranes and was terminated after 40 mins by filtration through polyethyleneimine (0.3%)-pretreated Whatman GF/B filters. The radioactivity of the filters was determined by liquid scintillation counting. Non-specific binding (<10 % of the total binding) was determined in the presence of 10 µM thioperamide. The radioactivity retained on the filters was determined by liquid scintillation counting.

Protein determinations were performed according to Bradford (1976).

H_3 receptor mediated inhibition of neurogenic contractions of the guinea-pig jejunum

The in vitro histamine H_3 receptor activity at the guinea-pig jejunum was determined as described by Vollinga *et al.* (1992). Male Dunkin-Hartley guinea-pigs (350-400 gram, Harlan CPB, Zeist, The Netherlands) were killed by cervical dislocation and the intestine was rapidly removed and kept in oxygenated (95% O_2/5% CO_2) Krebs buffer (composition in mM: NaCl 118, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25 and glucose 5.5). Whole jejunum segments (ca 2 cm) were mounted between two platinum electrodes (4 mm apart) in warm (37 °C) Krebs buffer under a load of 1 g. After 60 mins of equilibration the muscle was stimulated maximally (ca 15V) with a frequency of 0.1 Hz and a duration of 0.5 msec with rectangular wave electrical pulses (Grass Stimulator S-88, Grass Instruments Co., Quincy, USA). Contractions were recorded isotonically (Hugo Sachs TL-2/HF-modem, Hugo Sachs Elektronik, Hugstetten, Germany). After 30 mins of stimulation, a cumulative dose response curve for the H_3 receptor agonist (R)–α–methylhistamine was recorded. Antagonists were preincubated for 15 mins during the stimulation before the preparations were challenged again with (R)–α–methylhistamine. Maximally four dose response curves were recorded at one preparation.

H_3 receptor mediated inhibition of [^3H]-noradrenaline release from slices of rat brain

Based on a combination of the methods described by Schlicker *et al.* (1989) and Van der Werf *et al.* (1987), slices of the rat cerebral neocortex (0.3mm x 0.3mm) were incubated for 30 min in a Krebs-Ringer buffer (KRB, in mM: NaCl 118, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.4, $NaHCO_3$ 25, glucose 10, pH 7.4 at 37° C when gassed with O_2/CO_2 (95%/5%)) containing 5 μCi [^{3}H]-noradrenaline (specific activity=38 Ci/mmol, NEN). After washing 4 times with 10 ml of KRB, the slices were preperfused with KRB (containing 1 μM desipramine and 1 μM phentolamine), for 60 min at a flow of 0.3 ml/min under constant gassing (95% O_2, 5% CO_2). Subsequently, seven 10 min fractions were collected. Tritium overflow was evoked by a 200 seconds long electrical stimulation (rectangular pulses of 20 mA, 1ms, 0.3 Hz) during the second fraction (S_1) and the fifth fraction (S_2). Agonists were added immediately after the first stimulation (S_1). Antagonists were present from the beginning of the 60 minutes preperfusion and throughout the whole experiment. Finally, the slices were perfused for 20 min with 0.1N HCl to determine the total content of tritium.

Data analysis

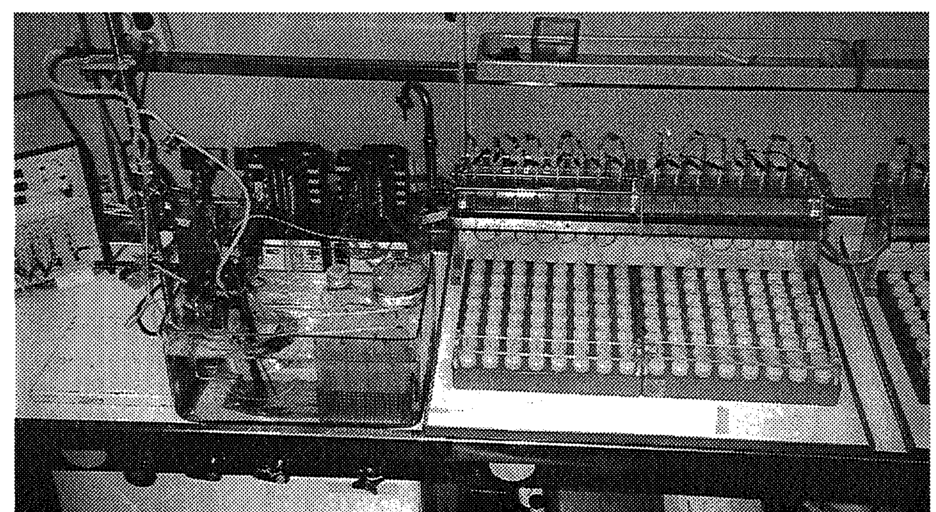
Results are given as mean±SEM of n experiments in duplicate (functional experiments) or of n experiments in triplicate (binding experiments). Displacement experiments were analysed by non-linear regression. The F-test was used to evaluate whether the displacement of [^{3}H]-N$^\alpha$-methylhistamine or [^{125}I]-iodophenpropit binding by the drugs under study was better fitted by a one- or two-site model.

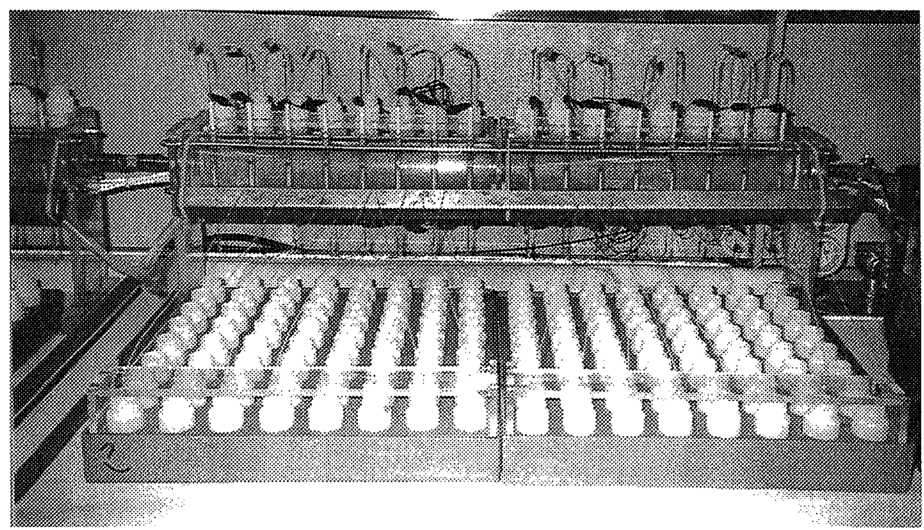
The stimulation-evoked tritium overflow from brain slices was calculated by substraction of the basal efflux from the total efflux during stimulation and the subsequent 13 minutes and was expressed as percentage of tissue tritium at the onset of stimulation. For quantification of the effect of H_3-agonists on the electrically evoked tritium overflow, the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1). The apparent pA_2 for the H_3 antagonists was calculated according to Furchgott (1972). Data obtained from functional assays were fitted to a sigmoidal curve using the program KaleidaGraph (Albelbeck Software).

Chemicals

N$^\alpha$–[methyl-^{3}H]-Methylhistamine dihydrochloride (78.9 Ci/mmol, NEN Boston MA), (-)-[ring-2,5,6-^{3}H] -noradrenaline base (38 Ci/mmol, NEN Netherlands); VUF compounds (all dihydrobromide), clobenpropit dihydrobromide, [^{125}I]-iodophenpropit

(specific activity 1950 Ci/mmol) (Menge *et al.*, 1992), thioperamide dihydrogen maleate (synthesized at the Department of Pharmacochemistry, Vrije Universiteit, Amsterdam, The Netherlands); desipramine hydrochloride (RBI); (R)–α–methylhistamine dihydrogen maleate (RBI); (S)–α–methylhistamine dihydrogen maleate (RBI); phentolamine hydrochloride gift from Ciba Geigy, B.V.; guanosine-5'-O-(3-thiotriphosphate) tetralithium salt (GTPγS, Sigma).





House-designed superfusion apparatus: **Upper**: Panoramic view, **Lower**: two of the 4 groups of 8 cells each.

Results

[^{125}I]-iodophenpropit binding experiments

In a study performed by Jansen *et al.* (1994) using [^{125}I]-iodophenpropit, saturation binding experiments to rat brain cortex membranes yielded a dissociation constant (K_d) of 0.57 ± 0.16 nM with a maximum number (Bmax) of binding sites of 268 ± 119 fmol/mg protein for this radiolabelled antagonist. Specific binding of 0.25 nM [^{125}I]-iodophenpropit to rat brain cortex membranes was monophasically displaced by the two immepip homologues (Table 1, Fig. 1).

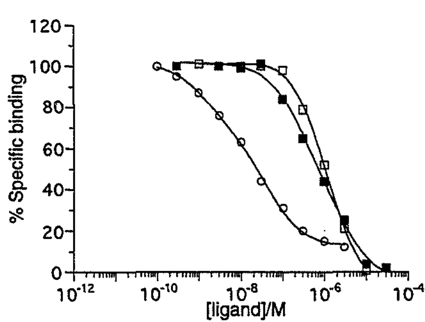


Figure 1 Inhibition of [^{125}I]-iodophenpropit binding (0.25 nM) by the H_3 receptor agonist immepip (open circles), VUF 4929 (filled squares) and VUF 4735 (open squares). Data are expressed as % specific binding determined with 0.3 μM thioperamide and represented 50%-60% of the total binding. The curves show fittings of a single representative experiment with triplicate determinations.

Immepip displaced the [^{125}I]-iodophenpropit binding (Table 1) according to a two site model. The presence of 10 μM GTPγS produced a steepening of the displacement curve of immepip and fitted better according to a one site model (Jansen *et al.*, 1994).

In the present study, both immepip homologues showed a moderate affinity for the binding of [^{125}I]-iodophenpropit with VUF 4929 showing slightly higher affinity (pK_i=6.5) than VUF 4735 (pK_i=6.0).

[^{3}H]-N$^{\alpha}$-methylhistamine binding experiments

In a study (Tedford *et al.*, 1995) using [^{3}H]-N$^{\alpha}$–methylhistamine, saturation binding experiments to rat brain cortex homogenates yielded a K_d value of 0.36±0.02 nM with a number of binding sites of 82±3 fmol/mg protein for this radioligand. Binding of 0.2 nM [^{3}H]-N$^{\alpha}$-methylhistamine to rat brain cortex membranes was displaced monophasically

by the tested immepip homologues (Table 1). Immepip was the most potent compound tested followed by VUF 4929 and VUF 4735.

H_3 receptor-mediated inhibition of neurogenic contractions of the guinea-pig jejunum

The electrically induced contractions of the guinea-pig jejunum are related to the release of acetylcholine and can be inhibited by the activation of H_3 receptors (see e.g. Vollinga *et al.*, 1992) It was previously shown that (R)-, (S)-α–methylhistamine and immepip effectively inhibited the electrically induced contractions with pD_2-values of 7.8 (Table 1, Vollinga *et al.*, 1994), 6.4 and 8.0, respectively (Vollinga *et al*, 1994; Leurs *et al.*, 1996). With none of the tested immepip homologues did we observe any inhibition of the neurogenic contractions (data not shown).

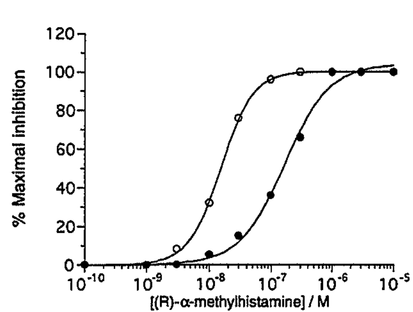


Figure 2 Inhibition of the electrically induced contractions of the guinea-pig jejunum by (R)-α-methylhistamine in the absence (open circles) and in the presence of 30 nM VUF 4929 (filled circles). The graph represents one typical experiment out of four independent experiments in duplicate.

In contrast, VUF 4735 (Table 1), and VUF 4929 (Fig. 2), shifted the dose-response curve of the (R)–α–methylhistamine-induced inhibition of the electrically induced contractions to the right. VUF 4929 was a potent antagonist in this system showing an apparent pA_2 value of 8.4 (Table 1).

H_3 receptor mediated inhibition of [^3H]-noradrenaline release

The electrically evoked tritium overflow from rat brain cortex slices preincubated with [^{3}H]-noradrenaline represents a quasi-physiological noradrenaline release (Kathmann *et al.*, 1993). This release is inhibited by the selective H_3 receptor agonists, (R) and (S)–α–methylhistamine (Fig.3) with pD_2 values of 7.8±0.1 (mean±SEM, n=10) and 6.2±0.1 (mean±SEM, n=3), respectively. The maximum inhibition obtained was 48±3% (mean±SEM, n=10). The dose response curve of (R)–α–methylhistamine was rightward shifted in the presence of 10 nM clobenpropit, yielding an apparent pA_2 of 9.4 for this compound (Fig. 3).

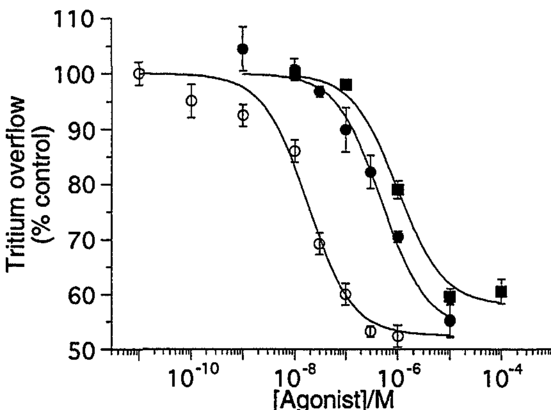


Figure 3 Inhibition of the electrical stimulated release of tritium from rat brain neocortex slices by (S)–α–methylhistamine (filled squares), (R)–α–methylhistamine in the absence (open circles) and in the presence of 10 nM clobenpropit (filled circles). Each point represents mean ± SEM of $3 \leq n \leq 10$ experiments performed in duplicate.

In a first series of experiments, the effect of the VUF compounds on the electrically evoked tritium overflow (expressed as S_2/S_1) was studied. The S_2/S_1 values, which were about unity in control experiments (not shown), were neither affected by VUF 4735 nor by VUF 4929 (Fig. 4A), thus indicating that the two immepip homologues are devoid of agonistic activity in this H_3 receptor model. In contrast, immepip inhibited the evoked overflow concentration-dependently (Fig. 4A). The maximum inhibitory effect obtained with immepip was 35±3 % (mean±SEM, n=3) (Fig. 4A, intrinsic activity (α)=0.8, pD_2=9.14±±0.09). The dose response curve of immepip was rightwards shifted in the presence of 10 nM clobenpropit yielding an apparent pA_2 value of 9.7 for clobenpropit (Fig. 4A).

In a second series of experiments the interaction of (R)–α–methylhistamine with 10 μM VUF 4735 and 3 μM VUF 4929 was studied. Both VUF compounds produced a rightward shift of the concentration-response curve of (R)–α–methylhistamine (Fig. 4B). Apparent pA_2 values, based on one concentration of antagonist were determined and are shown in Table 1.

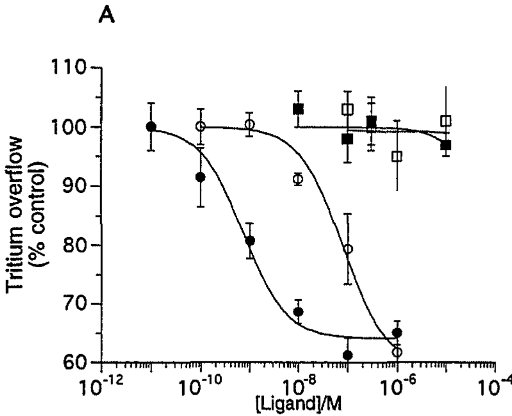
Table 1 Comparison of the H_3 receptor affinities and functional potencies of immepip and the immepip homologues VUF 4929 and VUF 4735.

				pKi	
Compound	(imidazolyl–$(CH_2)n$–piperidine; NH, N, NH)	**Neurogenic contractions**	**[^{3}H]NA release**	**[^{125}I]IPP binding**	**[^{3}H]NAMH binding**
VUF 4708 (immepip)	1	pD_2=8.0±0.03 (8)[a]	pD_2=9.14±0.09 (3)	8.5±0.1 / 6.0±0.1 (4)[b]	8.7±0.03 (3)
VUF 4929	2	pA_2=8.4±0.1 (4)	pA_2=7.14±0.02 (3)	6.5±0.1 (4)	7.7±0.1 (3)
VUF 4735	0	pA_2=6.2±0.1	pA_2=5.5±0.05 (3)	6.0±0.2 (4)	6.2±0.1 (3)

a) Vollinga *et al.* (1994); b) high and low affinities as determined by Jansen *et al.*, (1994)

Values shown represent means±s.e.m. of (N) experiments performed in triplicate (binding experiments) or duplicate (functional experiments).

A



B

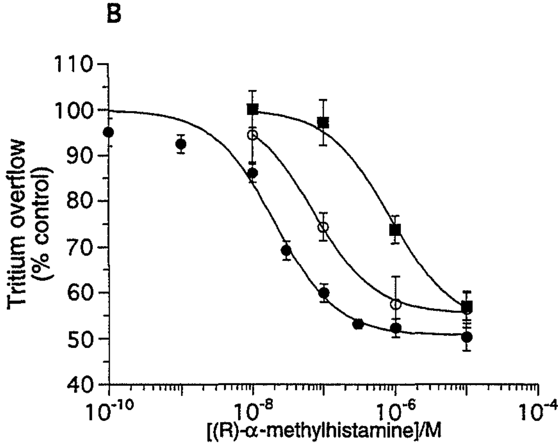


Figure 4 Inhibition of the electrical stimulated release of tritium from rat brain neocortex slices by (**A**) immepip (filled circles), immepip in the presence of 10 nM clobenpropit (open circles) VUF 4735 (filled squares) and VUF 4929 (open squares); (**B**) (R)–α–methylhistamine (filled circles) (R)–α–methylhistamine in the presence of 10 μM VUF 4735 (open circles) and (R)–α–methylhistamine in the presence 3 μM VUF 4929 (filled squares). Each point represents mean ± s.e.m. of $3 \leq n \leq 10$ experiments performed in duplicate.

Discussion

In the present study, the H3-receptor agonist immepip and two simple homologues were evaluated in several H3 receptor assays. These compounds only vary in the length

of the alkyl chain that separates the imidazole ring from the piperidine ring in which the basic amino function is incorporated (Table 1). Immepip has a methylene group linking the two rings, VUF 4929 has an ethylene group in that position whereas in VUF 4735 the imidazole is directly linked to the piperidine ring.

In all the assays immepip exhibited a potent H_3-receptor agonistic profile, whereas its "short" (VUF 4735) and "long" (VUF 4929) homologues behaved as antagonists. These results indicate the crucial role of the distance between the imidazole ring and the amino function. When incorporated in a piperidine ring, four carbon atoms between the imidazole and the basic amino function seem to be the ideal for H_3-agonism. Shortening (VUF 4735) or lengthening (VUF 4929) this chain completely abolishes H_3 receptor activation in both the guinea pig jejunum and the rat cerebral cortex models. This strict structural demands for H_3 receptor agonism makes immepip and its analogues useful tools for molecular modelling studies.

In the two H_3 receptor radioligand binding assays, using rat cerebral cortical membranes, immepip showed a relatively high affinity for the binding of the H_3 agonist $[^3H]$-NAMH (pK_i=8.7). Moreover, the H_3 receptor antagonist $[^{125}I]$IPP was displaced in a biphasic and GTPγS sensitive manner by immepip. These features are commonly observed for H_3 agonists (Leurs *et al.*, 1996) and in accordance with the H_3 agonistic effects of immepip. VUF 4735 exhibited virtually the same pK_i values in both binding assays (ca. 6.1). More difficult to interpret, however, were the results obtained for VUF 4929. This compound shows a 1.2 log unity higher affinity for the binding of $[^3H]$-NAMH than for $[^{125}I]$IPP binding. As no such agonist-like behaviour was detected for VUF 4929, this "discrepancy" remains unclear but confirms previous observations of pharmacological differences between the two radioligands (Jansen *et al.*, 1994; Kathmann *et al.*, 1993; West *et al.*, 1990) and may be explained by the existence of receptor subtypes.

At the guinea-pig jejunum immepip is a full agonist, yielding a pD_2 value of 8.0 (Vollinga *et al.*, 1994); this value is similar or slightly above the pD_2 value of the classical H_3-agonist (R)–α–methylhistamine (pD_2=7.8) (Vollinga *et al.*, 1994). In this test system, both "short" (VUF 4735) and "long" (VUF 4929) immepip analogues showed antagonistic activity at the H_3-receptor, parallely shifting the dose-response curve of (R)–α–methylhistamine rightwards. Apparent pA_2 values of 6.2 for VUF 4735 and 8.4 for VUF 4929 were obtained. This *in vitro* activity makes VUF 4929 a highly potent H_3-receptor antagonist comparable to thioperamide (pA_2=8.9; Vollinga *et al.*, 1992) and impentamine (pA_2=8.4, Leurs *et al.*, 1996).

Immepip and its two analogues were also tested for H_3-receptor activity in the rat brain. As previously reported by Schlicker *et al.* (1989), the $[^3H]$-noradrenaline release from rat cerebral cortex slices can be inhibited by the H_3 receptors agonists (R)- and (S)-α-methylhistamine in a stereoselective manner. This activity can be antagonised by

clobenpropit, yielding a pA_2 value of 9.4. This value closely corresponds with the H_3-antagonistic activity of clobenpropit in various other H_3 receptor models (Kathmann *et al.*, 1993; Leurs *et al.*, 1996). Immepip partially (α=0.8) inhibited the [^{3}H]-noradrenaline release from rat cerebral cortical slices for ca 35 % with a pD_2 value of 9.1, whereas full agonists like RAMH inhibited the release of [^{3}H]-noradrenaline for ca 50 % (pD_2=7.8). Also the response to immepip could be potently antagonised by clobenpropit (pA_2=9.7). Neither VUF 4735 nor VUF 4929 showed any agonistic activity in the rat brain. Both compounds antagonised the (R)–α–methylhistamine induced inhibition of the electrically evoked release of [^{3}H]-noradrenaline. The pA_2 values obtained for these compounds in this test system were 5.5 for VUF 4735 and 7.1 for VUF 4929. These values differ considerably from the pA_2 values obtained at the guinea pig jejunum. Moreover, a much lower potency was detected for immepip in the guinea pig jejunum (pD_2=8.0) than in the rat brain (pD_2=9.1). This last finding could be interpreted as a more efficient coupling of the central H_3 receptor to its effector as compared to the peripheral system. Nevertheless, virtually no difference was detected for the potency of RAMH and SAMH in both functional assays making an interpretation based on receptor coupling efficiency unsatisfactory. Moreover, one would not expect for immepip partial agonism (α=0.8) in the rat brain and full agonism in the guinea pig jejunum. Species differences, however, could still account for these differences. Taking into consideration what is observed with classical H_3 receptor ligands like RAMH and thioperamide, and in view of the increasing experimental evidences reported by other research groups (Schlicker *et al.*, 1996; Schworer *et al.*, 1994) and by our group (Leurs *et al.*, 1996), one is, nevertheless, further tempted to accept the existence of H_3-receptor subtypes.

The strongest evidence for H_3 receptor heterogeneity is, in our opinion, obtained from the different H_3-receptor functional assays. At present, the results from the different radioligand binding assays are difficult to match. As observed previously (Leurs *et al.*, 1996) for H_3-receptor antagonists the affinities obtained from [^{3}H]-NAMH binding assay match quite well the H_3-receptor potency at the guinea pig jejunum. In contrast, the K_i values calculated from the binding of [^{125}I]IPP show considerable discrepancies between the ones obtained from the binding of [^{3}H]NAMH.

In conclusion, comparing our results with those published by Leurs *et al.* (1996) a critical role for the distance between the imidazole ring and the basic amino function in histamine H_3-receptor ligands is again observed. When the amino group is part of a piperidine ring, as in the immepip molecule, a four carbon chain seems to be the ideal for H_3-agonism, a shorter (VUF 4735) or a longer (VUF 4929) spacer will lead to total loss of agonistic activity. In contrast, for the aliphatic compounds, possessing a higher flexibility, less stringent demands for the alkyl spacer between the imidazole and the basic amino function have been found for H_3 agonism in the brain (Leurs *et al.*, 1996).

Evaluation of the immepip homologues in different H3 receptor assay revealed important differences; such differences would not be expected to occur if the same receptor subtype was present in the tissues studied. With this study, we have given further support to the existence of histamine H3-receptor subtypes for which several ligands show clear discriminative behaviour. Due to the growing interest on H3 ligands as putative therapeutic agents in several CNS disorders (see chapter 1), detailed information with respect to H3-receptor heterogeneity is mandatory.

Immepip and its ligands might become attractive compounds for drug development as they do not possess toxic (iso)thiourea moieties as thioperamide and clobenpropit do (Van der Goot *et al.*, 1992) and because they seem to be able to discriminate between central and peripheral H3-receptors.

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Chapter 4

[^{3}H]-THIOPERAMIDE AS A RADIOLIGAND FOR THE HISTAMINE H_3 RECEPTOR IN RAT CEREBRAL CORTEX

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Summary

1 The purpose of the present study was to characterise the binding of the histamine H_3 receptor antagonist [^{3}H]-thioperamide to rat cerebral cortical membranes.

2 The binding of [^{3}H]-thioperamide to rat cerebral cortical membranes reached equilibrium after incubation with [^{3}H]-thioperamide after 8-10 hours at 4°C. Equilibrium was maintained for up to 18 hours of incubation. Addition of 1 μM (R)-α-methylhistamine rapidly caused dissociation of [^{3}H]-thioperamide from its binding sites. Based on these kinetic experiments a dissociation constant of 0.3 nM was obtained for [^{3}H]-thioperamide.

3 Saturation experiments with [^{3}H]-thioperamide using 1 μM (R)-α-methylhistamine to define non-specific binding were best analysed according to a single site model. A dissociation constant (K_D) of 0.80±0.06 nM (n=3) and a maximal number of binding sites (B_{max}) of 73±20 fmol/mg protein (n=3) were obtained for the binding of [^{3}H]-thioperamide to rat cerebral cortical membranes.

4 Saturation experiments with [^{3}H]-thioperamide using 0.3 μM iodophenpropit to define non-specific binding were best analysed according to a two site model. For the high affinity [^{3}H]-thioperamide site a K_D value of 1.1±0.3 nM (n=3) and B_{max} value of 162±108 fmol/mg protein (n=3) were obtained whereas K_D and B_{max} values for the low affinity site were 96±19 nM and 4346±3092 fmol/mg protein (n=3), respectively.

5 Using 5 nM [^{3}H]-thioperamide, the binding was hardly displaced by H_3 agonists within concentration ranges expected to bind to the histamine H_3 receptor. Under these conditions, [^{3}H]-thioperamide binding was fully displaced by various H_3-antagonists. Yet, most H_3 antagonists showed K_i values different from those expected for the histamine H_3 receptor.

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6 Using 0.3 nM [^{3}H]-thioperamide, 50-60% of the total binding was potently displaced by the H_3 agonists histamine, (R)-α–methylhistamine, (S)-α–methylhistamine, imetit and immepip. Displacement of the binding of 0.3 nM [^{3}H]-thioperamide binding exhibited clear stereoselectivity for the R and S isomers of α–methylhistamine.

7 Binding of 0.3 nM [^{3}H]-thioperamide was completely displaced by several H_3 antagonists (thioperamide, iodophenpropit, iodoproxyfan, and burimamide) and biphasic displacement curves were obtained; the K_i values for the high affinity site corresponded well with the expected values for the H_3 receptor. Antagonists fully displaced the binding of 5 nM [^{3}H]-thioperamide with affinities comparable to the low affinity site found at 0.3 nM [^{3}H]-thioperamide.

8 Ondansetron and haloperidol did not displace binding of 5 nM [^{3}H]-thioperamide at concentrations at which the former are known to bind to 5-HT_3 or σ receptors, respectively. On the other hand, nonselective cytochrome P_{450} inhibitors displaced the binding of 5 nM [^{3}H]-thioperamide from both rat cerebral cortical membranes and rat liver microsomes.

9 It is concluded that the histamine H_3 antagonist, [^{3}H]-thioperamide, can be used as a radioligand to study the histamine H_3 receptor in rat brain provided that subnanomolar concentrations are used in displacement studies. Moreover, the specific binding should be defined with an H_3 agonist, since most H_3 antagonists share with [^{3}H]-thioperamide a low affinity, high density, non-H_3 receptor binding site(s) in rat brain. The latter is likely due to binding to cytochrome P_{450} isoenzymes.

Introduction

The histamine H_3 receptor was originally identified as a presynaptic receptor that regulated the synthesis and release of histamine in the CNS (Arrang *et al.*, 1987; Arrang *et al.*, 1983; Van der Werf *et al.*, 1987). This receptor is also present as an heteroreceptor on non-histaminergic neurones and is known to regulate the release of several other neurotransmitters such as 5-hydroxytryptamine (Alves Rodrigues *et al.*, 1995; Schlicker *et al.*, 1988), noradrenaline (Schlicker *et al.*, 1989), dopamine (Schlicker *et al.*, 1993) and acetylcholine (Clapham & Kilpatrick, 1992) in both the central and peripheral nervous systems (Barnes *et al.*, 1993; Bertaccini *et al.*, 1991). Concomitant with the initial pharmacological definition of this new histamine receptor subtype Arrang *et al.* (1987) described (R)-α–methylhistamine and thioperamide as the first selective H_3 agonist and antagonist, respectively. Although many other potent and

selective H_3-receptor agonists and antagonists have since been introduced (Leurs *et al.*, 1995a), the early availibility of thioperamide made this compound one of the most used and best characterised H_3 antagonists. In H_3 receptor binding studies thioperamide shows an affinity for the H_3 receptor in the low nanomolar range (Arrang *et al.*, 1990; Jansen *et al.*, 1994). However, interactions with 5-HT_3 and σ receptors have been observed at higher concentrations (Leurs *et al.*, 1995b). Furthermore, thioperamide has been shown to inhibit steroidogenesis by an interaction with cytochrome P450 isoenzymes (Labella *et al.*, 1992).

Acting as an H_3 antagonist thioperamide has been reported to affect central and peripheral functions *in vivo*. In the CNS of laboratory animals thioperamide shows anxiolytic (Imaizumi & Onodera, 1993) and anticonvulsant (Yokoyama *et al.*, 1993) properties; it also increases locomotor activity (Sakai *et al.*, 1991), improves learning and memory skills (Meguro *et al.*, 1995), increases wakefulness (Monti, 1993), inhibits amphetamine-induced hyperactivity (Clapham & Kilpatrick, 1994), and decreases food intake (Oohara *et al.*, 1994; Sakata *et al.*, 1994). Based on these pharmacological activities, there has been increasing interest in the development of H_3 antagonists as potential therapeutical tools particularly for CNS disorders.

Despite the early availability and high affinity of thioperamide for the H_3 receptor, its labelling and use as a radioligand for the H_3 receptor was reported as unsuccessful (Yanai *et al.*, 1994). Yanai *et al.* (1994) briefly reported on the use of the related (S)-[^{3}H]-methylthioperamide as a radioligand for the histamine H_3 receptor. Although early results regarding its affinity and autoradiographic tissue distribution in the rat brain appear promising, the pharmacological definition of its binding site(s) is not yet clear. For example, H_3 receptor density observed in the rat forebrain (Yanai *et al.*, 1994) was 5 fold higher than values reported for [^{3}H]-(R)-α-methylhistamine binding.

In the present study we established experimental conditions under which [^{3}H]-thioperamide (Figure 1) can be used as a radioligand to study the histamine H_3 receptor *in vitro*. New insights on the definition of H_3 receptor specific binding using radiolabelled H_3 antagonists are also presented. Despite its low specific activity, when compared to most of the labelled H_3 antagonists described (Jansen *et al.*, 1994; Ligneau *et al.*, 1994; Yanai *et al.*, 1994), [^{3}H]-thioperamide can be used as radioligand to study the histamine H_3 receptor in the rat brain. As such, thioperamide, a brain penetrating compound, may be of importance for the development of radioligands with short half-lives for use in PET studies in CNS disorders where the histamine H_3 receptor is suspected to be involved (Alves Rodrigues *et al.*, 1995; Onodera *et al.*, 1994; Smith *et al.*, 1994; Yokoyama *et al.*, 1994).

Figure 1 Structure of [^{3}H]-thioperamide. Asterisks indicate the positions labeled with tritium.

Methods

Synthesis of [^{3}H]-thioperamide

Cyclohexylisothiocyanate (2.5 mg) was dissolved in 250 µl of ethanol. [^{3}H]4-(4-piperidyl) imidazole (50 mCi in 0.6 ml ethanol, 6 Ci/mmol) was added to this solution together with triethylamine (2.2 mg). The mixture was heated in a sealed vial at 55-60°C for five hours. The mixture was separated on a preparative C-18 HPLC column (Novo Nordisk A/S, 16 x 250 mm, 7 µM), using as eluent a mixture of triethylamine (0.2%, pH 6.0 using phosphoric acid) and acetonitrile 70:30 (v:v). The flow rate was 6.0 ml/min. The collected fractions (R_t=30-35 min) of the product were concentrated and extracted with dichloromethane. The organic layer was dried ($MgSO_4$), filtered, and solvents were eliminated under vacuum to yield a yellow oil. The final product was stored at -20°C in methanol.

HPLC analysis of the radiolabelled product was performed using a S5 Phenyl column (250 x 4.6 mm, 5 µm, Phase Sep) using as eluent a mixture of triethylamine (0.2%, pH 6.0 adjusted with phosphoric acid) and acetonitrile 85:15 (v:v). The flow rate was 2.0 ml/min. UV absortion (215 nm) and radioactivity (Radiomatic/Canberra Flo-One beta detector A-200) were monitored.

The radiochemical yield was 45%. The final product had a specific activity of 6.0 Ci/mmol (determined by HPLC with non-labelled thioperamide as a reference standard). The radiochemical purity was higher than 98%.

Preparation of membranes from rat cerebral cortex

Male Wistar rats (200-220 g) were decapitated and the brains were rapidly removed. Whole cortices were dissected and homogenised in 10 volumes (v:w) of ice-cold 50 mM Tris-HCl buffer (containing 5 mM $MgCl_2$, 145 mM NaCl, pH 7.4 at 4°C) using an

Ultra-Turrax blender and a Potter-Elvhjem homogeñeizer. This homogenate was centrifuged at 800 g for 10 min. The pellet was discarded and the supernatant was centrifuged at 40,000 g for 40 min. The resulting pellet was rinsed twice under the same conditions. The final pellet was resuspended in 1.5 volumes (v:w) of the Tris-HCl buffer described above and stored at -80°C until the day of the experiment when it was diluted 2.5-fold (v:w) in the same solution.

[^{3}H]-thioperamide binding assays

[^{3}H]-thioperamide binding assays were carried out based on the procedure described by Jansen *et al.* (1994) for the binding of [^{125}I]-iodophenpropit. Kinetic (using 0.3 nM [^{3}H]-thioperamide, Figure 2), saturation (using either 1 μM R-(α)-methylhistamine, Figure 3B; or 0.3 μM iodophenpropit, Figure 3A) , and displacement experiments (using both 0.3 nM and 5 nM of [^{3}H]-thioperamide; Figures 4 and 5) were performed at 4 °C in 50 mM Tris-HCl buffer described above in a total incubation volume of 0.25 or 0.5 ml, using polyethylene tubes. Determinations were performed in duplicate. Drugs were prepared in the same buffer. Rat cerebral cortical membranes and rat liver microsomes (Jefcoate, 1978), previously prepared and kept at -80° C, were incubated for 14 hours to reach equilibrium. Incubations were started by the addition of membranes and were terminated by the addition of 3 ml of ice-cold Tris-HCl buffer (pH 7.4, at 4°C) followed immediately by filtration through Whatman GF/B filters using a Brandel filtration apparatus. Filter binding was less than 1% of total radioactivity added. After filtration of the membranes filters were washed once with 3 ml of ice-cold Tris-HCl buffer then transferred to vials. Scintillation fluid was added and the radioactivity bound to the filters was measured in a Wallac beta scintillation counter. Samples were left to equilibrate for 24 hours before counting for 5 minutes per sample.The standard error of the mean of the dpm counted did not exceed 5% both when the experiments were performed in triplicate or in duplicate. Counting efficiency was 60%.

Protein concentrations were determined using the Bio-Rad Protein Assay kit (based on Bradford, 1976). Bovine serum albumin was used as standard.

Data analysis

Saturation and competition binding experiments were evaluated on a Macintosh computer using the non-linear curve fitting programme LIGAND (Munson & Rodbard, 1980). With the aid of this programme binding curves were, respectively, fitted (unweighted) to a one and two independent sites models. The improvement of the fit for

each model with additional parameters was evaluated based on the 'extra sum of squares' principal (Draper & Smith, 1966), taking $p<0.05$ to be significant.

Chemicals

[^{3}H]4-(4-piperidyl) imidazole with a specific activity of 6 Ci/mmol was obtained from Amersham and used without further purification. Cyclohexylisothiocyanate was synthesised at Novo Nordisk A/S. All other reagents and solvents used in the synthesis of [^{3}H]-thioperamide were of analytical grade.

For the binding studies the following drugs were used: thioperamide maleate, iodophenpropit dihydrobromide, imetit dihydrobromide, immepip dihydrobromide, (all synthesised at the Department of Pharmacochemistry, Vrije Universiteit, Amsterdam, The Netherlands); iodoproxyfan, (R)-α-methylhistamine maleate (gifts from Dr. W. Schunack, Berlin), (S)-α-methylhistamine dihydrobromide (Cookson Chemicals), burimamide (gift from Smith Kline Beecham), haloperidol (RBI, Natick, U.S.A.), ondansetron (gift from Solvay Duphar), histamine dihydrochloride (Sigma), imidazole (Merck), SKF 525A hydrochloride (RBI), metyrapone (Aldrich-Chemie).

Results

Time course of the [^{3}H]-thioperamide binding to rat cortical membranes

Our initial binding studies with [^{3}H]-thioperamide were performed at 37° C as described in the methods of Jansen *et al.* (1994). However, a very slow association (>10 hours to reach equilibirium) of 5 nM [^{3}H]-thioperamide to rat brain cortical membanes was observed. In parallel experiments with 600 μg/ml membrane protein we observed at 5 nM [^{3}H]-thioperamide 2921 ± 152 and 1342 ± 43 dpm total binding (mean ± s.d., n = 3) at 4° and 37° C, respectively. At 37° C almost no total binding of 0.3 nM [^{3}H]-thioperamide could be detected (100 ± 20 dpm), whereas at 4 °C 1430 ± 50 dpm total binding was found. Therefore, our studies were thereafter performed at 4° C. At this temperature, binding of 0.3 nM [^{3}H]-thioperamide to rat brain cortical membranes reached equilibrium after 8-10 hours (Figure 2) resulting in an association constant (K_{on}) of 0.2 ± 0.1 10^9 $M^{-1}.min^{-1}$ (mean±s.d., n=3). The binding of [^{3}H]-thioperamide was readily reversible with the addition of 1 μM (R)-α–methylhistamine which displaced 80-85% of the specific binding with a dissociation constant (K_{off}) of 0.065 ± 0.056 min^{-1} (mean±s.d., n=3). Based on these kinetic data the K_d value ($K_{off/on}$) of [^{3}H]-thioperamide was calculated to be 0.3 ± 0.4 nM (mean ± sd, n = 3).

At 4° C the binding of 0.3 nM (inset Figure 2) and 5 nM [^{3}H]-thioperamide (data not shown) to rat cortical membranes increased linearly with increasing concentrations of

protein up to 400 μg/ml. Based on these characteristics, saturation and displacement experiments were performed using an incubation period of 14 hours, at 4° C, with 200-400 μg/ml of protein.

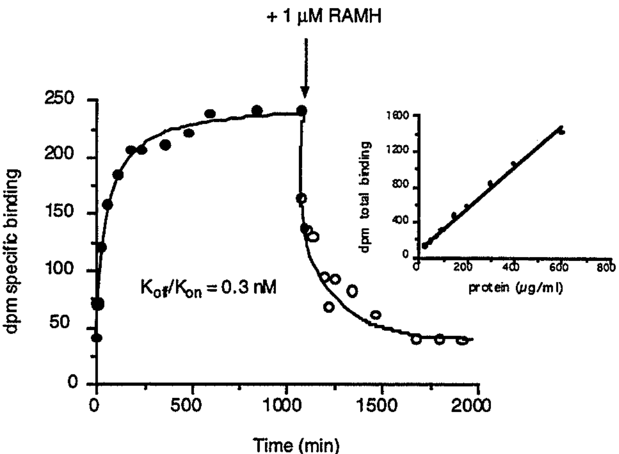
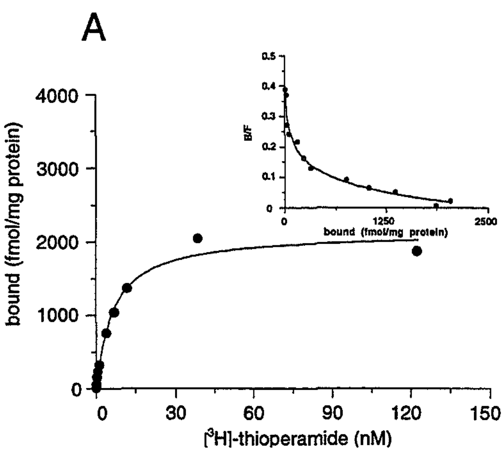


Figure 2 Association and dissociation curves of the [^{3}H]-thioperamide binding to rat brain cortical membranes. Membranes (50 μg/tube) were incubated at 4° C with 0.3 nM [^{3}H]-thioperamide in a final volume of 250 μl. Non-specific binding was measured in the presence of 1 μM (R)-α-methylhistamine. The specific binding at equilibrium represented 50-60% of the total binding. For the dissociation of [^{3}H]-thioperamide binding, (R)-α-methylhistamine (1 μM) was added, after a 18 hours incubation. One typical experiment of three is shown. Inset shows the binding of 0.3 nM [^{3}H]-thioperamide at different protein concentrations. A typical experiment performed in triplicate is shown. Similar data was obtained in two other independent experiments.

[^{3}H]-thioperamide saturation binding experiments

Incubation of rat cerebral cortical membranes with increasing concentrations of [^{3}H]-thioperamide showed binding to be saturable. When specific binding was determined using 0.3 μM of iodophenpropit, biphasic Scatchard plots were obtained (Figure 3A); analysis of the [^{3}H]-thioperamide saturation curves (0.03-120 nM) revealed two populations of binding sites (Table1). When specific binding was determined using 1 μM (R)-α-methylhistamine (Figure 3B) a linear Scatchard plot was obtained and [^{3}H]-thioperamide binding (0.03-7 nM) was best fitted according to a single site model (Table 1). Specific binding represented 50 to 55 % of the total binding at [^{3}H]-thioperamide concentrations below 0.5 nM. Specific binding decreased significantly when the

concentration of [^{3}H]-thioperamide increased. Non-specific binding was a linear function regardless of the way it was defined.



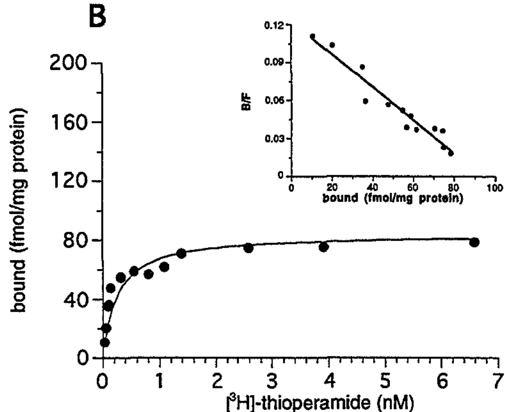


Figure 3 Saturation binding of [^{3}H]-thioperamide to membranes (200 μg/tube) from rat cerebral cortex. The insets show the transformation of the data into Scatchard plots. (A) The non-specific binding was defined in the presence of 0.3 μM iodophenpropit. (B) The non-specific binding was defined in the presence of 1 μM (R)-α-methylhistamine. Results shown are from one representative experiment of three performed in duplicate.

Table 1 Dissociation constants (K_D) and number of specific binding sites (B_{max}) for the binding of [^{3}H]-thioperamide to membranes of rat brain cortex. The radioligand binding studies were performed as described in Methods. Data represent mean±s.d. of three independent experiments performed in duplicate.

Non-specific binding defined with:	**K_D** (high) **(nM)**	**B_{max}** (high) **(fmol/mg protein)**	**K_D** (low) **(nM)**	**B_{max}** (low) **(fmol/mg protein)**
0.3 μM iodophenpropit	1.1±0.3	162±108	96±19	4346± 3092
1 μM (R)-α-methylhistamine	0.8±0.1	73±20	-	-

Displacement curves using H_3-antagonists

Total binding of [^{3}H]-thioperamide to rat cerebral cortical membranes was fully displaced by H_3 antagonists. Figure 4 shows displacement of 0.3 and 5 nM [^{3}H]-thioperamide by the two H_3 antagonists iodophenpropit (Figure 4A) and iodoproxyfan (Figure 4B).

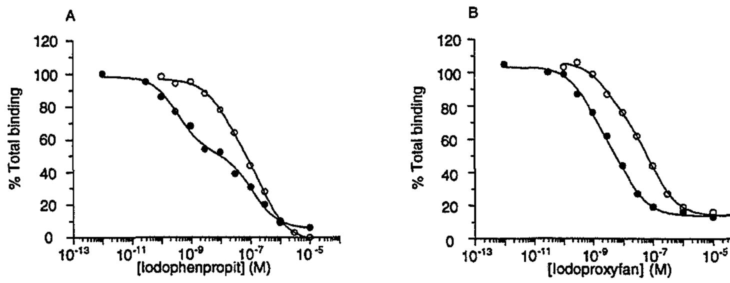


Figure 4 Displacement of the total binding of 0.3 nM (filled circles) and 5 nM (opened circles) [^{3}H]-thioperamide to rat brain cortical membranes (100 μg/tube) at 4°C by iodophenpropit (A) or iodoproxyfan (B). Data represent a typical experiment of at least three independent experiments performed in duplicate.

When 0.3 nM [^{3}H]-thioperamide was used in competition assays, the H_3-receptor antagonists distinguished between high and low affinity components. K_i values obtained for the high affinity site were consistent with those reported for the H_3-receptor

(table 2). Using 5 nM [^{3}H]-thioperamide, the displacement curves were all fitted best to a single site model. The K_i values obtained, under these conditions, differed from those expected for the H_3 receptor and were mostly comparable to K_i values for the low affinity site found at 0.3 nM (Table 2).

Table 2 Affinity of different ligands towards [^{3}H]-thioperamide binding sites on rat cerebral cortex membranes.

K_i *high* / K_i *low* (*nM*)			
Ligand	*0.3 nM* *[^{125}I]-iodophenpropit*	*0.3 nM* *[^{3}H]-thioperamide*	*5 nM* *[^{3}H]-thioperamide*
H_3-Agonists:			
Histamine	38±10 / 2500±600[a)]	143±29	N.D.
(R)-α-methylhistamine	3.5±1.2 / 1200±300[a)]	9±3 / 180±15	N.D.
(S)-α-methylhistamine	230±97 / 9500±180[a)]	250±156	N.D.
Imetit	2.7±1 / 40000±12000[a)]	8.1±5.6	N.D.
Immepip	2.7±1 / 1000±200[a)]	2.8±0.7	N.D.
H_3-antagonists:			
Iodophenpropit	0.97±0.06[a)]	0.36±0.14 / 80±19	9.5±1.0
Thioperamide	4.3±1.6[a)]	0.66±0.64 / 14±9	20±12
Burimamide	18±9 / 725±392[a)]	11.4±4.1 / 454±93	103±13
Iodoproxyfan	2.4±0.2	0.33±0.16 / 6.3±1.0	6±2

a) Determined by Jansen *et al.* (1994)
The compounds that show high and low affinity K_i values fitted best to a two site model ($p<0.05$). Values are given as mean±s.d. of at least three independent experiments performed in duplicate. N.D.; not determined.

Displacement curves using H_3-agonists

Figure 5 shows the displacement of the binding of 5 nM and 0.3 nM [^{3}H]-thioperamide by (R)-α-methylhistamine, histamine, and (S)-α-methylhistamine. When 5 nM [^{3}H]-thioperamide was used, less than 15% of the total binding was displaced by the tested H_3 agonists applying a concentration range expected to bind to the H_3 receptor. Yet, even under these conditions it was possible to observe stereoselectivity of

the (R) and (S) isomers of α-methylhistamine (Figure 5A). Nevertheless, the small amount of binding displaced did not allow proper fittings of the agonist curves.

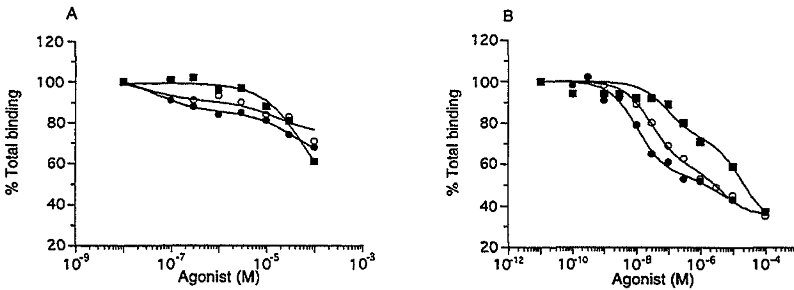


Figure 5 Displacement of the total binding of 5 nM (A) and 0.3 nM (B) [^{3}H]-thioperamide to rat brain cortical membranes (100 μg/tube) at 4°C by (R)-α-methylhistamine (filled circles), (S)-α-methylhistamine (filled squares), and histamine (opened circles). Data represent one of at least three independent experiments performed in duplicate.

On the other hand, when experiments were performed with 0.3 nM [^{3}H]-thioperamide up to 60% of total binding was displaced by the H_3 agonists (Figure 5B). Also in these experiments, stereoselectivity between the (R) and (S) isomers of α-methylhistamine was evident up to micromolar concentrations. K_i values obtained for these compounds and for other well established H_3 agonists (imetit and immepip) are shown in table 2. Biphasic curves were obtained only for the displacement of 0.3 nM [^{3}H]-thioperamide by (R)-α-methylhistamine. Nevertheless, this displacement was not affected by the presence of 10 μM GTPγS (data not shown). For the other agonists LIGAND was unable to discriminate two independent sites, although at the higher agonist concentrations the agonists started to displace a seconde site.

Displacement of [^{3}H]-thioperamide by other ligands

Thioperamide displays high to moderate affinity for 5-HT_3 and for σ receptors (Leurs *et al.*, 1995b). Hence ondansetron, a 5-HT_3 selective ligand, and haloperidol, which shows high affinity for the σ receptor, were tested in displacement assays. Neither haloperidol (K_i>10 μM, n=3) nor ondansetron (K_i=10 μM, n=3) displaced the binding of 5 nM of [^{3}H]-thioperamide at concentrations selective for the σ or 5-HT_3-receptors, respectively.

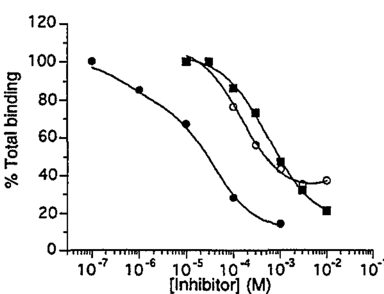


Figure 6 Displacement of the total binding of 5 nM [^{3}H]-thioperamide to rat brain cortical membranes (100 µg/tube) at 4°C by imidazole (filled circles), metyrapone (open circles), and SKF 525A (filled squares). Data represent one of at least three independent experiments performed in duplicate.

Imidazole, metyrapone, and SKF 525A are known to inhibit cytochrome P450 isoenzymes nonselectively (Halpert *et al.*, 1994). These compounds displaced up to 80% of the total binding of 5 nM [^{3}H]-thioperamide in rat cerebral cortex membranes (Figure 6) at concentrations known to inhibit binding to cytochrome P450 isoenzymes (Halpert *et al.*, 1994). The IC_{50} values obtained for imidazole, metyrapone, and SKF 525A were, respectively: 11±1 µM (mean±s.d., n=3), 522±92 µM (mean±s.d., n=3), and 481±18 µM (mean±s.d., n=3).

Finally, we investigated the binding of 5 nM [^{3}H]-thioperamide to rat liver microsomes. Total binding of 5 nM [^{3}H]-thioperamide to 950 µg/ml rat liver microsomal proteins amounted 61014±431 dpm. This binding was inhibited by 12.1±6.3 %; 88.9±0.4 %; 93.1±0.1 %; 90.5±0.1 % (mean ± sd, n = 2) by respectively, 1 µM RAMH, 100 µM imidazole, 1 mM metyrapone, and 1 mM SKF 525A.

Discussion

Until recently, the highly selective H_3 agonists, [^{3}H]-(R)-α–methylhistamine and [^{3}H]-N^{α}–methylhistamine, were the only tools available to label the H_3 receptor and they have been of great use in the elucidation of the pharmacological characteristics of the H_3 receptor (Arrang *et al.*, 1988; West *et al.*, 1990). Nevertheless, the binding properties of radiolabelled agonists are known to be complex and often difficult to interpret (West *et al.*, 1990; Clark & Hill, 1995). In an attempt to solve some of the difficulties related to the use of labelled agonists, new H_3 antagonists were synthesised, radiolabelled and pharmacologically characterised. The first radiolabelled H_3 antagonist was [^{125}I]-iodophenpropit (Menge *et al.*, 1992) an iodinated analogue of clobenpropit, the most potent H_3 antagonist available to date (Leurs *et al.*, 1995a). This radioligand is

suitable for characterisation histamine H_3 receptors in rat cerebral cortex (Jansen *et al.*, 1992; Jansen *et al.*, 1994). Yet, competition experiments with the two H_3 antagonists, burimamide and dimaprit, showed biphasic displacement curves suggesting that this radioligand interacts with two binding sites (Jansen *et al.*, 1994). [^{125}I]-iodoproxyfan, another iodinated H_3 antagonist, was used to label H_3 receptors in rat striatum (Ligneau *et al.*, 1994). However, agonists displaced a maximum of 60% of the total binding whereas antagonists fully displaced the binding of [^{125}I]-iodoproxyfan. Apparently [^{125}I]-iodoproxyfan labels an additional binding site with high affinity. The labelling of this secondary site(s) by [^{125}I]-iodoproxyfan was potently inhibited by H_3 antagonists, sometimes resulting in complex displacement curves with Hill coefficients significantly higher than one (Ligneau *et al.*, 1994).

In the present study we describe the synthesis of the radiolabeled H_3 antagonist [^{3}H]-thioperamide as well as the pharmacological characterization of its binding to rat cerebral cortical membranes. Under the experimental conditions used, [^{3}H]-thioperamide binding was saturable and reversible. When saturation experiments were performed (0.01-7 nM [^{3}H]-thioperamide) using the H_3-agonist (R)-α-methylhistamine to define non-specific binding, linear Scatchard plots were obtained consistent with labelling of a single class of binding sites (Table 1). When competition experiments were performed using 0.3 nM [^{3}H]-thioperamide, H_3 agonists displaced up to 60% of the total binding. Stereoselectivity for the (R) and (S) isomers of the H_3 agonist α-methylhistamine was observed within the concentration interval expected to bind to the H_3 receptor. In contrast to previous studies with [^{125}I]-iodophenpropit at 37° C (Jansen *et al.*, 1994) agonist displacement of [^{3}H]-thioperamide was not affected by GTPγS at 4°C. Since recently Clark & Hill (1996) presented conclusive evidence for the interaction of the H_3 receptor with pertussis toxin-sensitive G proteins in the rat cerebral cortex, the lack of GTPγS effect in the present study is likely due to differences in the thermodynamic/thermokinetic behaviour of G-protein coupled receptors. For example, it has been shown that for agonist binding to β_2-adrenergic receptors the high affinity dissociation constant decreases much more with decreasing temperature than the low affinity constant (Miklavc *et al.*, 1990).

At higher concentrations of [^{3}H]-thioperamide H_3 agonists also displaced the [^{3}H]-thioperamide binding, but stereoselectivity for the isomers of α–methylhistamine was almost totally lost. We, therefore, consider 60% of the total binding of 0.3 nM [^{3}H]-thioperamide to rat brain cortical membranes to represent the H_3 receptor. Displacement studies with several H_3 antagonists confirmed this conclusion. For these ligands displacement studies with 0.3 nM [^{3}H]-thioperamide resulted in biphasic displacement curves. The K_i values for the high affinity [^{3}H]-thioperamide binding site (approximately 50-60% of total binding) corresponded well with the known affinities for the H_3 receptor (Jansen *et al.*, 1994) for the tested H_3 antagonists.

When 5 nM of [^{3}H]-thioperamide was used only a small part (<15%) of the total binding was displaced by H_3 agonists. Yet, under these conditions, the H_3 antagonists fully displaced [^{3}H]-thioperamide binding monophasically at concentrations which mostly did not correspond with their observed affinities for the H_3 receptor. Our findings indicate that at 5 nM [^{3}H]-thioperamide binds mostly to a secondary, non-H_3, binding site(s). Furthermore, the H_3 antagonists we tested showed moderate to high affinity for this secondary site(s), albeit at concentrations more than 10 fold higher than their affinities for the H_3 receptor. The relatively high affinity of iodophenpropit for the secondary [^{3}H]-thioperamide site(s) (K_i=80 nM) appears to account for the observed curvilinear Scatchard plots of the [^{3}H]-thioperamide saturation curves, when iodophenpropit was used to define the non-specific binding (Figure 3A). Although the estimates of the density of the binding obtained were relatively inaccurate for the secondary site(s) (Table 1), it is evident that this low affinity [^{3}H]-thioperamide binding site(s) is present in large excess over the H_3 receptor binding site.

Previously Leurs *et al.*, (1995b) extensively characterised the receptor selectivity of iodophenpropit and thioperamide. In those radioligand binding studies displacement with thioperamide showed that the latter had relatively high affinities for the 5-HT_3 (120±30 nM) and σ (180±90 nM) receptors. Present results with the selective σ ligand haloperidol and the selective 5-HT_3 ligand ondansetron indicated that these two receptors do not contribute significantly to the total binding of [^{3}H]-thioperamide.

Thioperamide has also been shown to interact with the cytochrome P_{450} isoenzymes (Labella *et al.*, 1992). Imidazole, metyrapone, and SKF 525A, all nonselective inhibitors of cytochrome P_{450} isoenzymes (Halpert *et al.*, 1994), showed almost full displacement of the binding of 5 nM [^{3}H]-thioperamide in rat cerebral cortical membranes strongly indicating that those enzymes may be a major source for the non-H_3 receptor component [^{3}H]-thioperamide binding. Since cytochrome P_{450} enzymes are present in relatively high amounts in the brain cortex (74 pmol/mg protein) (Ravindranath, 1995) the binding to cytochrome P_{450} isoenzymes may explain the high density of the binding observed at higher concentrations of [^{3}H]-thioperamide (Table 1, Figure 3). This suggestion is supported by our observation that [^{3}H]-thioperamide also binds to rat liver microsomes. Labelling of a cytochrome P_{450} isoenzyme has also been reported to complicate histamine H_1 receptor binding studies with [^{3}H]-mepyramine (Fukui *et al.*, 1995; Leurs *et al.*, 1989; Leurs *et al.*, 1990).

Considering the results obtained from this study, the following picture regarding the available radiolabelled H_3 antagonists emerges. [^{125}I]-iodophenpropit (Jansen *et al.*, 1994) can be considered a suitable radioligand to label H_3 receptors as it markedly discriminates between high and low affinity binding sites of H_3 antagonists in the rat cerebral cortex (selectivity ratio 222). Yet, it would be advisable to use agonists to define non-specific binding, in saturation studies in particular where the non-specific

binding increases markedly with increasing concentrations of radioligand. [^{125}I]-iodoproxyfan (Ligneau *et al.*, 1994) should be used with more care when labelling the H_3 receptor. This compound does not discriminate well between high and low affinity binding sites of H_3 antagonists in the rat cerebral cortex (selectivity ratio 19). This observation is consistent with the finding that only 60% of the total binding of this ligand to rat striatal membranes is displaced by H_3 agonists while several antagonists fully displace the binding of [^{125}I]-iodoproxyfan (Ligneau *et al.*, 1994). [^{3}H]-(S)-methylthioperamide (Yanai *et al.*, 1994) binding was characterised only with (R) and (S) methylhistamine, and thioperamide. Moreover, non-specific binding was defined using 10 μM of thioperamide. In view of our recent results this procedure appears to be inadequate for the selective labelling the H_3 receptor. Indeed, Yanai *et al.* (1994) reported relatively high densities of (S)-[^{3}H]-methylthioperamide binding in the rat brain. In various peripheral tissues, such as the liver and the lung, where the H_3 receptor is not expected to be present (Korte *et al.*, 1990) the density of the binding sites for (S)-[^{3}H]-methylthioperamide was even higher (Yanai *et al.*, 1994). This can be due to binding of this radioligand to cytochrome P_{450} isoenzymes present in high densities in these tissues (Ravindranath, 1995).

In conclusion, [^{3}H]-thioperamide binds to rat brain cortical membranes in a saturable and reversible manner showing high and low affinity components. The high affinity site is likely to represent the histamine H_3 receptor as the binding is displaced by (R) and (S)-α–methylhistamine in a stereoselective manner and by several other H_3 ligands with a pharmacological profile of the H_3 receptor. At nanomolar concentrations binding of [^{3}H]-thioperamide to a low affinity non-H_3 receptor binding site(s) increases steeply, reaching, at saturation, a density 30 fold higher than the number of H_3 receptors. Also other H_3 antagonists we tested show high affinity for the non-H_3 receptor sites. Therefore, based on this study, it would be judicious to use H_3 agonists rather than H_3 antagonists to delineate specific (and non-specific) binding of radioactively labelled H_3 antagonists.

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Chapter 5

PHARMACOLOGICAL CHARACTERISATION OF THE HISTAMINE H3 RECEPTOR IN THE RAT HIPPOCAMPUS.

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Summary

Saturation binding experiments with the H3 receptor antagonist [^{125}I]-iodophenpropit revealed labelling of a single class of binding sites, in rat hippocampus membranes. A dissociation constant value of 0.33±0.07 nM (mean±SEM, n=3) and a maximal number of binding sites of 125±25 fmol/mg protein (mean±SEM, n=3) were obtained. [^{125}I]-iodophenpropit (0.25 nM) binding to rat hippocampal membranes was readily displaced by histamine H3 receptor agonists and antagonists, with K_i values within the range expected to bind to the histamine H3 receptor and comparable to the values obtained for the H3 receptor present in the rat brain cortex. Stereoselectivity between the (R) and (S) enantiomers of the H3 receptor agonist α-methylhistamine was observed. In the presence of GTPγS a rightwards shift ocurred for the high affinity site of the binding of (R)-α-methylhistamine. Up to a concentration of 1 μM, (R)-α-methylhistamine displaced up to 30% of the total binding of [^{125}I]-iodophenpropit whereas H3-antagonists displaced 50-60% of the bound [^{125}I]-iodophenpropit. This observation suggests the presence of an additional, non-H3 receptor, component of the binding of H3-antagonists in the rat hippocampus.

Functionally, an H3 receptor mediated inhibition of the [^{3}H]-noradrenaline release was detected in the rat hippocampus. A maximal inhibition of 42.4±6.3 % (mean±SEM, n=16) was observed. The pD_2 values obtained for (R) and (S)-α-methylhistamine were, respectively, 7.6±0.1 (mean±SEM, n=7), and 6.5±0.04 (mean±SEM, n=6). For the H3-antagonists thioperamide and burimamide pA_2 values of 8.0±0.3 (mean±SEM, n=3) and 7.3±0.2 (mean±SEM, n=6), respectively, were obtained.

Introduction

In the CNS, histaminergic cell bodies are exclusively found in the tuberomammillary nucleus of the posterior hypothalamus (Panula *et al.*, 1990). In contrast, histamine synthesising nerve terminals project throughout the whole brain and to all regions of the hippocampal formation (Panula *et al.*, 1990; Schwartz *et al.*, 1990). This brain structure is directly involved in synaptic plasticity and cognitive functions (Haas *et al.*, 1995; Zola-

Morgan *et al.*, 1986). The involvement of histamine in memory linked events has long been suggested (De Almeida & Izquierdo, 1988) but has gradually gained interest only during the recent years. Already in 1983 Haas *et al.* showed that histamine reduces a calcium-dependent K^+ conductance in hippocampal pyramidal cells. This effect was shown to be mediated by receptors known to stimulate adenylyl cyclase, such as the histamine H_2 receptor (Haas & Konnerth, 1983). In view of the current knowledge, it is clear that such effects increase depolarisation and calcium entry and, thereby, facilitate long term potentiation (LTP) induction and maintenance (Haas *et al.*, 1995). Moreover, independently and almost simultaneously, two research groups reported on the histamine potentiation of NMDA-mediated synaptic transmission in the hippocampus (Bekkers, 1993; Vorobjev *et al.*, 1993) via an interaction with a polyamine regulatory binding site on the NMDA channel. By selectively enhancing NMDA mediated neurotransmission, histamine potentiates processes in which NMDA currents are involved such as the triggering of LTP (Bekkers, 1993; Vorobjev *et al.*, 1993; Zola-Morgan *et al.*, 1986). Consequently, physiological or pathological conditions that deplete histamine in the brain might lead to a reduced ability to trigger LTP and, therefore, memory loss (Bekkers, 1993, 1996).

The presynaptic histamine H_3 autoreceptor is a regulatory unit for the synthesis and release of histamine in the CNS (Arrang *et al.*, 1983; Arrang *et al.*, 1987; Van der Werf *et al.*, 1987). The histamine H_3 receptor not only regulates the release of histamine from histaminergic nerve terminals but is also present on non-histaminergic nerve terminals, where it acts as a heteroreceptor modulator of the release of other biogenic amines such as acetylcholine (Arrang *et al.*, 1995; Clapham & Kilpatrick, 1992), serotonin, noradrenaline, and dopamine in the CNS (Alves Rodrigues *et al.*, 1995; Leurs *et al.*, 1996; Schlicker *et al.*, 1988; Schlicker *et al.*, 1993; Schlicker *et al.*, 1989; Schlicker *et al.*, 1994).

The enthorinal cortex is a constitutive portion of the hippocampal formation responsible for an unidirectional excitatory projection from the cerebral cortex into the dentate gyrus and from there to all other fields of the hippocampus (Amaral & Witter, 1989). Deterioration of cholinergic transmission in the enthorinal cortex is often linked to memory loss (Bierer *et al.*, 1995). This observation together with the reported H_3 receptor modulation of the release of acetylcholine in the enthorinal cortex (Arrang *et al.*, 1995; Clapham & Kilpatrick, 1992) strongly support the hypothesis of the involvement of the histaminergic system, and of the H_3 receptor in particular, in hippocampal memory formation. Furthermore, using behavioural studies in rodents, several authors have reported on the cognitive enhancing properties of the H_3 receptor antagonist thioperamide, both under normal physiological conditions (Barnes *et al.*, 1993; Miyazaki *et al.*, 1995) and after scopolamine-induced learning deficits (Shichi *et al.*, 1995) or accelerated senescence (Meguro *et al.*, 1995).

The presence of histamine H3 receptors in the hippocampus of the rodent (Cumming *et al.*, 1991; Jansen *et al.*, 1994; Pollard *et al.*, 1993; Yanai *et al.*, 1994) and of the primate (Martinez-Mir *et al.*, 1990) has previously been visualised using autoradiographic techniques. Nevertheless, a pharmacological characterisation of the hippocampal H3 receptor has, so far, not been described. Following up the previous work by our group in the rat brain cortex (Jansen *et al.*, 1994) and in view of the increasing evidence for the involvement of histamine in hippocampal cognitive function, we pharmacologically characterised the histamine H3 receptor in the rat brain hippocampus.

Methods

Preparation of rat brain membranes

Male Wistar rats (200-220 g, Harlan C.P.B., Zeist, The Netherlands) were decapitated and the brains were rapidly removed. The hippocampi were dissected and homogenised in 10 volumes (v:w) of ice-cold 50 mM Tris-HCl buffer (with 5 mM $MgCl_2$, 145 mM NaCl, pH 7.4 at 4°C) using a Ultra-Turrax blender and a Potter-Elvehjem homogeniser. This homogenate was centrifuged at 800 g for 10 min. The pellet was discarded and the supernatant centrifuged at 40,000 g during 40 min. The resulting pellet was rinsed twice under the same conditions. The final pellet was resuspended in 1.5 volumes (v:w) of the above described Tris-HCl buffer and stored at -80°C until the day of the experiment when it was diluted 2.5 (v:w) times in the same solution.

[^{125}I]-iodophenpropit binding assays

[^{125}I]-iodophenpropit binding assays were carried out based on the procedure described by Jansen *et al.* (1994). Saturation, and competition binding experiments of [^{125}I]-iodophenpropit (1950 Ci/mmol) were performed at 37° C in 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 145 mM NaCl (pH 7.4 at 37°C) in a total incubation volume of 0.5 ml (0.25 ml, in saturation binding experiments), using polyethylene tubes. Determinations were performed in triplicate. Compounds were diluted in buffer. Rat brain membranes, previously prepared and kept at -80° C, were incubated for 60 min to reach equilibrium. In saturation experiments membranes were incubated with [^{125}I]-iodophenpropit in final concentrations ranging from 0.075 nM to 3 nM. In these assays, the non-specific binding was defined using 1 μM of (R)-α-methylhistamine. Incubations were started upon addition of 100 μl (50 μl, in saturation binding experiments) membranes (30-80 μg of protein per tube) and were terminated by the addition of 3 ml of ice-cold Tris-HCl buffer (pH 7.4, at 4°C) immediately followed by filtration through Whatman GF/C filters using a Brandel filtration apparatus. Filters were

pretreated for at least 2 hours with 0.3% polyetheleneimine, reducing filter binding to less than 1% of the total radioactivity added. After filtration of the membranes the filters were washed twice with 3 ml of ice-cold Tris-HCl buffer. The amount of radioactivity bound to the membranes was not reduced by repetition of the washing procedure. The radioactivity bound to the filters was measured by an LKB gamma counter. Protein concentrations were determined using the Bio-Rad Protein Assay kit (based on Bradford, 1976). Bovine serum albumin was used as standard.

Functional assays

Based on a combination of the methods described by Schlicker *et al.* (1989) and Van der Werf *et al.* (1987) slices of the rat cerebral neocortex or hippocampus (0.3mm x 0.3mm) were incubated for 30 min in a Krebs-Ringer buffer (KRB) (in mM: NaCl 118, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.4, $NaHCO_3$ 25, glucose 10, pH 7.4 at 37° C when gassed with O_2/CO_2 (95%/5%)) containing 5 µCi [^{3}H]-noradrenaline (specific activity=38 Ci/mmol, NEN). After washing 4 times with 10 ml of KRB, the slices were preperfused with KRB (containing 1 µM desipramine and 1 µM phentolamine), for 60 min at a flow of 0.3 ml/min under constant gassing (95% O_2, 5% CO_2). Subsequently, seven 10 min fractions were collected, tritium overflow was evoked by a 200 seconds long electrical stimulation (rectangular pulses of 20 mA, 1ms, 0.3 Hz) during the second fraction (S_1) and the fifth fraction (S_2). Agonists were added right after the first stimulation (S_1). Antagonists were present from the beginning of the 60 minutes preperfusion and throughout the whole experiment. Finally, the slices were perfused for 20 min with 0.1N HCl to determine the total content of tritium.

Data analysis

Saturation and competition binding experiments were evaluated on a Macintosh computer using the non-linear curve fitting programme LIGAND (Munson & Rodbard, 1980). With the aid of this programme binding curves were, respectively, fitted (unweighted) to a one and two independent sites models. The improvement of the fit for each model with additional parameters was evaluated based on the 'extra sum of squares' principal (Draper & Smith, 1966), taking $p<0.05$ to be significant.

The stimulation-evoked tritium overflow was calculated by substraction of the basal efflux from the total efflux during stimulation and the subsequent 13 minutes and was expressed as percentage of tissue tritium at the onset of stimulation. For quantification of the effect of H_3-agonists on the electrically evoked tritium overflow, the ratio of the overflow evoked by S_2 over that evoked by S_1 Was determined (S_2/S_1). The apparent pA_2 for the H_3 antagonists was calculated according to Furchgott (1972). Data obtained

from functional assays were fitted to a sigmoidal curve using the program KaleidaGraph (Albelbeck Software).

Chemicals

[^{125}I]-Iodophenpropit was labelled to a specific activity of 1950 Ci/mmol as described by Menge *et al,* (1992). [^{3}H]-Noradrenaline (specific activity=38 Ci/mmol) was purchased from NEN, Netherlands. Thioperamide maleate, and iodophenpropit dihydrobromide were synthesised at the Department of Pharmacochemistry, Vrije Universiteit, Amsterdam, The Netherlands; (R)-α–methylhistamine maleate, and (S)–α–methylhistamine dihydrobromide were purchased from Cookson Chemicals), burimamide was a gift from Smith Kline Beecham, polyethylenimine was purchased from Aldrich-Chemie, guanosine 5'-o-(3-thio)triphosphate (GTPγS) from Sigma, desipramine hydrochloride was purchased from RBI and phentolamine hydrochloride was a gift from Ciba Geigy. Iodoproxyfan was kindly provided by Dr. W. Schunack (Berlin).

Results

[^{125}I]-iodophenpropit saturation binding assays

Specific binding (determined in the presence of 1 μM (R)-α–methylhistamine) of [^{125}I]-iodophenpropit (0.075-3 nM) to rat hippocampal membranes was saturable (Figure 1) and yielded linear Scatchard plots (inset Figure 1) revealing binding of the radioligand to a single class of sites. Computer analysis of the binding curves showed a dissociation constant (K_D) of 0.33±0.07 nM (mean±SEM, n=3) and a maximal number of binding sites of 125±25 fmol/mg protein for [^{125}I]-iodophenpropit (mean±SEM, n=3). Non-specific binding increased linearly with the concentration of [^{125}I]-iodophenpropit. Specific binding represented 30-35% of the total binding of 0.25 nM [^{125}I]-iodophenpropit, decreasing rapidly with increasing concentrations of [^{125}I]-iodophenpropit.

Competition binding assays

A stereoselective binding of the (R) and (S) enantiomers of the H_3-receptor agonist α-methylhistamine was established (Figure 2A, table 1). (R)-α-methylhistamine showed a biphasic displacement curve of the binding of [^{125}I]-iodophenpropit; the fitting programme LIGAND could not clearly distinguish between high and low affinity sites of the binding of (S)-α-methylhistamine. Upon addition of GTPγS (10 μM) the competition

binding curve of (R)–α–methylhistamine showed a rightward shift, indicating coupling of the H3-receptor to a G-protein in the hippocampus; this curve fitted best to a one site fit model (Figure 2A, Table 1).

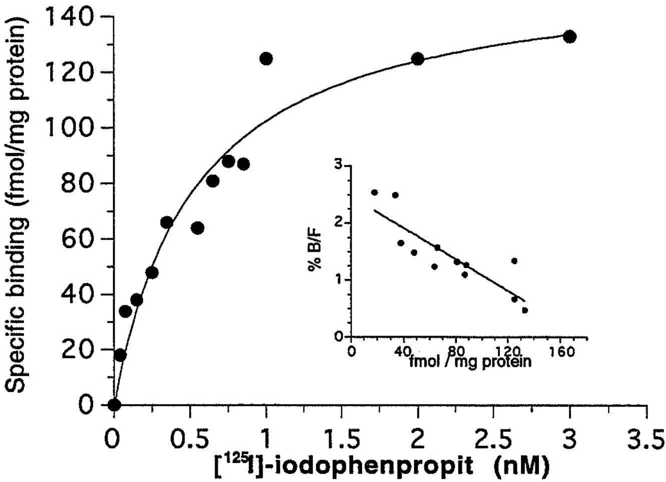


Figure 1 Saturation binding of [^{125}I]-iodophenpropit to membranes from rat hippocampus. The inset shows the transformation of the data into a Scatchard plot. The nonspecific binding was defined in the presence of 1 μM (R)-α-methylhistamine. Results shown are from one representative experiment of three performed in triplicate.

Surprisingly, and in contrast with observations from the rat cerebral cortex (Jansen *et al.*, 1994), only ca. 30% of the total binding of 0.25 nM [^{125}I]-iodophenpropit was displaced by the H3-receptor agonists (R) and (S) α-methylhistamine within the concentration range expected to bind to the H3 receptor (Figures 2A, 2C); whereas the binding of 0.25 nM [^{125}I]-iodophenpropit displaced by the H3-antagonist thioperamide represented 50-60% of the total binding (Figure 2C). This component of the binding was equally displaced by the other H3-receptor antagonists, burimamide, and iodoproxyfan, within the concentration range expected to bind to the H3 receptor (Figure 2B). With the exception of burimamide, all the H3 receptor antagonists tested fitted best to a one site model (P>0.05). The K_i values obtained for the several H3 receptor antagonists are shown on table 1.

Table 1 Affinities (Ki values) of several H3-receptor ligands for [^{125}I]-iodophenpropit binding sites on rat hippocampus and cerebral cortex membranes. Potencies of H3-agonists (pD_2) and antagonists (pA_2) from inhibition of the electrically evoked release of [^{3}H]-noradrenaline.

	Ki (hippocampus) (nM)	*Ki (cortex) (nM)*	*pD_2/pA_2 (cortex)*	*pD_2/pA_2 (hippocampus)*
H3-receptor antagonists:				
Thioperamide	6.5±1.2 (7)	0.93±0.22 (3)[a)]	8.0±0.2 (3)	8.0±0.3 (3)
Iodoproxyfan	2.4±0.2 (6)	2.4±0.2 (3)	N.D.	N.D.
Burimamide	7.7±0.8 / 2,103±777	18±9/725±138 (8)[a)]	7.05[b)]	7.3±0.2 (6)
H3-receptor agonists:				
(R)-α-methylhistamine	1.3±0.6 / 270±200 (8)	3.5±0.6 / 1,200±150 (4)[a)]	7.8±0.1 (10)	7.6±0.1 (7)
(R)-α-methylhistamine+GTPγS	137±34 (4)	100±50 (4)[a)]	N.D.	N.D.
(S)-α-methylhistamine	1,270±261 (7)	230±49 / 9,500±900 (4)[a)]	6.2±0.1 (3)	6.0±0.1 (6)

a) extracted from Jansen *et al.* (1994); b) extracted from Schlicker *et al.* (1989)
The compounds that show high and low affinity Ki values fitted best to a two site model ($P<0.05$). Values are given as mean±s.e.m. of (N) independent experiments. N.D.; not determined.

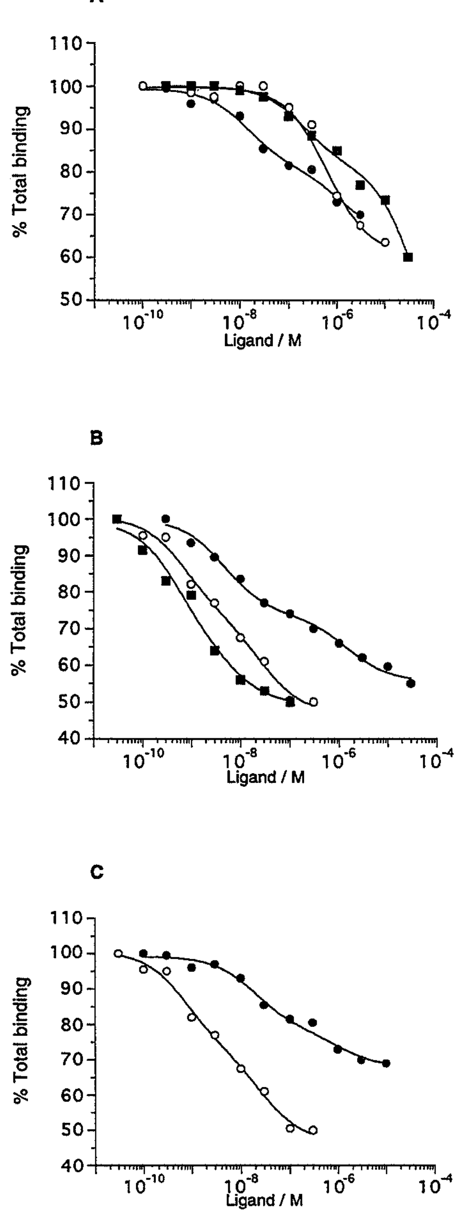
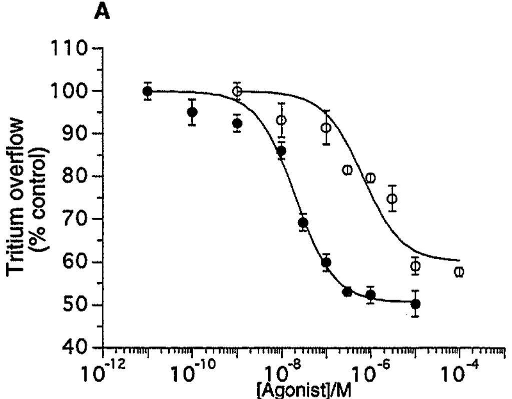


Figure 2 Displacement of the total binding of 0.25 nM [^{125}I]-iodophenpropit by (**A**) (R)-α-methylhistamine (filled circles), (R)-α-methylhistamine + 10μM GTPγS (open circles), and (S)-α-methylhistamine (filled squares); (**B**) iodophenpropit (filled squares), iodoproxyfan (open circles) and burimamide (filled circles); (**C**) thioperamide (open circles), (R)-α-methylhistamine (filled circles). Data represent a typical experiment of at least three independent experiments performed in triplicate.

Functional studies

The electrically evoked tritium overflow was inhibited in a concentration dependent and in a stereoselective manner by the H3-receptor agonists (R) and (S)-α-methylhistamine both in the cortex (Figure 3A) and in the hippocampus (Figure 3B).



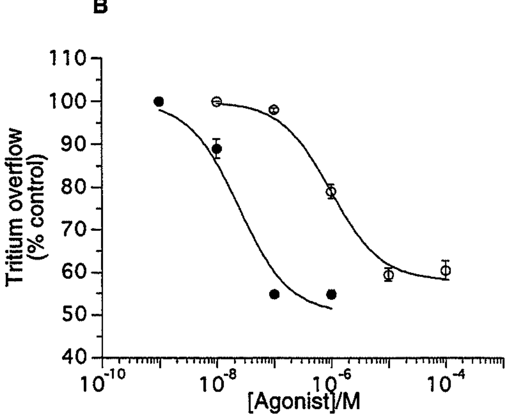


Figure 3 Effect of (R)-α-methylhistamine (filled circles) and (S)-α-methylhistamine (open circles) on the electrically stimulated tritium overflow from superfused rat brain slices preincubated with [^{3}H]-noradrenaline in the (**A**) Cortex: , (**B**) hippocampus. Each point represents mean±SEM of 3-7 experiments performed in duplicate.

Under control conditions the ratio S_2/S_1 accounted 0.91±0.02 (mean±SEM, n=3) in the cerebral cortex and 0.89±0.02 (mean±SEM, n=16), in the hippocampus. A maximum inhibition of 48.3±1.6% (mean±SEM, n=3) and 42.4±2.3% (mean±SEM, n=16) by (R)-α-methylhistamine was obtained in the rat brain cortex and hippocampus, respectively. Thioperamide (10^{-7}M) and burimamide (10^{-6}M) shifted the (R)-α-methylhistamine concentration response curve to the right in the hippocampus (Figure 4). It should be mentioned, however, that burimamide displayed a slight non-competitive behaviour as the maximal inhibition obtained for (R)-α-methylhistamine in the presence of 1 μM burimamide was only 37.7%±1.5% (mean±SEM, n=6). Neither thioperamide (S_2/S_1=0.97±0.02; mean±SEM, n=3) nor burimamide (S_2/S_1=0.87±0.1; mean±SEM, n=6), by themselves, affected the electrically evoked release of [^{3}H]-noradrenaline in the hippocampus. The pD_2 values obtained for (R) and (S) α-methylhistamine and the pA_2 values obtained for thioperamide and burimamide are depicted on table 1.

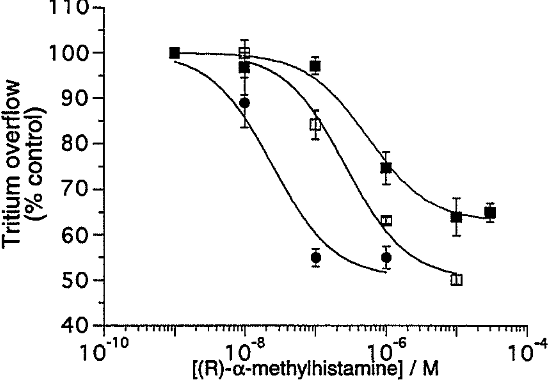


Figure 4 Effect of (R)-α-methylhistamine (filled circles), (R)-α-methylhistamine in the presence of 10^{-7}M thioperamide (open squares) and (R)-α-methylhistamine in the presence of 10^{-6}M burimamide (filled squares) on the electrically stimulated tritium overflow from superfused rat brain slices preincubated with [^{3}H]-noradrenaline. Each point represents mean±SEM of 3-7 experiments performed in duplicate.

Discussion

Following up the characterisation of the rat cerebral cortical histamine H3 receptor using [^{125}I]-iodophenpropit (Jansen *et al.*, 1994), and in view of the recent results regarding the possible link between the histamine H3 receptor and memory formation, we characterised, in the present study, the rat hippocampal histamine H3 receptor. We used

the H3-receptor antagonist [^{125}I]-iodophenpropit in receptor binding assays, and the H3 receptor modulation of the electrically stimulated release of [^{3}H]-noradrenaline as a functional assay.

In agreement with the data obtained in the rat cortex by (Jansen *et al.*, 1994), saturation binding assays using hippocampal membranes revealed labelling of a single class of high affinity [^{125}I]-iodophenpropit binding sites. The comparison of B_{max} values between the hippocampus and the cerebral cortex, revealed a clear difference. The receptor density in the hippocampus (B_{max}=125 fmol/mg protein) showed a value approximately two fold lower compared to the value reported by (Jansen *et al.*, 1994) for the rat cerebral cortex (B_{max}=272 fmol/mg protein). This observation confirms data previously established from autoradiographic studies in the rat brain in which the density of the histamine H3 receptor in the hippocampus is lower than that of the cerebral cortex (Cumming *et al.*, 1991; Ligneau *et al.*, 1994; Pollard *et al.*, 1993; Yanai *et al.*, 1994).

Nevertheless, other factors have accounted for the difference in the density of receptors detected between this and autoradiographic studies. In the present work, only approximately 30% of the binding of 0.25 nM [^{125}I]-iodophenpropit was displaced by the H3 receptor agonists (R) and (S)-α-methylhistamine (Figure 2A) while 50-60% was displaced by the H3 receptor antagonists studied. The same was seen by Ligneau *et al.*, (1994) who characterised the striatal H3 receptor using [^{125}I]-iodoproxyfan. Moreover, in a study using quantitative storage phosphor autoradiography, a similar situation was observed for the binding of [^{125}I]-iodophenpropit in the rat hippocampus by Jansen *et al.* (unpublished data). Our interpretation for this apparent discrepancy is the presence of a high affinity non-H3 receptor component of the binding of H3 receptor antagonists which is not present in the binding of the H3 receptor agonists. This component seems to be present in higher densities in the hippocampus relatively to the cortex, since Jansen *et al.*, (1994) described an almost equal level of displacement of the binding of 0.25 nM [^{125}I]-iodophenpropit by (R)-α-methylhistamine and thioperamide in the cortex. Comparable data were obtained in chapter 4 using the labelled H3-antagonist [^{3}H]-thioperamide, for which we observed that 90% of the binding of 5 nM [^{3}H]-thioperamide to rat cerebral cortical membranes is not to the histamine H3 receptor but to a high density low affinity site, probably representing cytochrome P450 isoenzymes (Alves-Rodrigues *et al.*, 1996). Hence, in view of these results, we recommend that saturation binding assays with radiolabelled H3 antagonists are performed using 1 μM (R)–α–methylhistamine to define the non-specific binding.

When competition binding studies in the hippocampus are concerned, both in the hippocampus and in the cortex of the rat brain a clear stereoselectivity was observed for the (R) and (S) enantiomers of the H3 receptor agonist α-methylhistamine. GTPγS shifts for (R)–α–methylhistamine were obtained in both brain areas confirming (Zweig *et al.*, 1992) the coupling of histamine H3 receptors to G-proteins. It was rather difficult,

however, to determine the H_3-receptor affinity of antagonists. Similar to what was seen by Ligneau *et al.* (1994) in the rat striatum, several H_3 antagonists did not show a clear distinction between the H_3 and a non-H_3 receptor components of the binding of radiolabelled H_3-antagonists (this study, Alves-Rodrigues *et al.*, 1996; Ligneau *et al.*, 1994). Although we know that, under our experimental conditions [^{125}I]-iodophenpropit is labelling more than one site in the hippocampus, the fitting programme LIGAND did not analyse the displacement curves of the H_3-antagonists according to a two-site model. Only for burimamide a clear distinction between the two sites was found. One would expect from these observations that the K_i values for the H_3-receptor obtained for the displacement of [^{125}I]-iodophenpropit by H_3 antagonists would be underestimated. Nevertheless, the values calculated from these curves are in agreement with their functional potency (Table 1), and with the values obtained by Jansen *et al.* (1994) in the cerebral cortex, indicating, just by chance, similar affinities of the H_3 antagonists studied for the H_3 receptor and the non-H_3 receptor component of the binding in the rat hippocampus.

In the second part of our study we characterised, for the first time, a functionally active H_3-receptor in the rat hippocampus. The H_3-receptor mediated inhibition of the electrically evoked release of [^{3}H]-noradrenaline is comparable with the one observed in the rat cerebral cortex (Schlicker *et al.*, 1989). No significant differences were detected between the levels of maximum inhibition of the electrically evoked release of [^{3}H]-noradrenaline or with the control ratio S_2/S_1 between the hippocampus the cerebral cortex. Both the pD_2 values obtained for (R) and (S)-α-methylhistamine, and the pA_2 values for thioperamide and burimamide showed similar values in the two cerebral areas studied (Table 1; Schlicker *et al.*, 1989). Sara (1989) published on a noradrenergic-cholinergic interaction and its possible role in senile dementia associated memory disfunction. Experimental observations in the hippocampus supported the author's hypothesis that when there is a reduction in the cholinergic function, a corresponding decrease in noradrenaline release (by clonidine) reduces the noradrenergic inhibitory influence on acetylcholine release in terminals of spared cholinergic neurons.

In summary, we report on the characterisation of the histamine H_3 receptor in the rat brain hippocampus. In accordance with autoradiography studies, a lower receptor density was obtained in the rat hippocampus as compared to the rat brain cortex. There were no significant differences among the affinities or the potencies of the H_3-ligands studied in the hippocampus and cortex (Jansen *et al.*, 1994). Intriguingly, in the hippocampus, H_3-antagonists bind to a a non-H_3 receptor component of the binding, which was not detected in the cerebral cortex (Jansen *et al.*, 1994). In chapter 4, we have suggested that this component is one or more P450 isoenzymes. The difference between the two brain areas may be due to the higher abundance of P450 isoenzymes in the hippocampus compared to the total cerebral cortex (Ravindranath, 1995). This observation should be

carefully addressed in studies in which receptor densities are of relevant importance. Bearing this in mind, we have delineated conditions to study the histamine H3 receptor in the hippocampus and the potential changes in its pharmacological and/or physiological properties in relation to memory and learning. These studies will be of major importance for obtaining detailed insight into the potential therapeutic application of H3-receptor ligands (for review see Leurs *et al.*, 1995).

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Chapter 6

AGE-RELATED CHANGES IN THE DENSITY AND FUNCTIONALITY OF HISTAMINE H3-RECEPTORS IN THE RAT BRAIN.

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Summary

Age-dependent changes in the histamine H_3-receptor density were assessed in rat brain cortex, striatum, and hippocampus using radioligand binding studies with the H_3-receptor antagonist [^{125}I]-iodophenpropit. Brain tissue obtained from F_1 rats of 2 months (young) and 28 months (old) of age were examined. A 95% increase in the density of H_3-receptors in the rat hippocampus was observed with ageing. In contrast, a decrease of 35% ocurred in the cerebral cortex and virtually no changes in the H_3-density were detected in the striatum of old rats. In the three regions examined, no significant age-related differences were observed for the K_d values of the radiolabelled H_3-antagonist [^{125}I]-iodophenpropit.

Using a sensitive HPLC technique with fluorescence detection, we also evaluated the histamine content of nine discrete brain regions. Histamine levels showed a significant (23%) increase in the hypothalamus of old rats, whereas no age-dependent changes were measured in the histamine levels of the other brain regions examined (including the hippocampus, the striatum and the cortex).

In functional studies, the histamine H_3 receptor mediated inhibition of the electrically evoked release of [^{3}H]-noradrenaline in both the hippocampus and the cortex showed signifcant age-related decreases in the maximal inhibition. The pD_2 value of (R)-α-methylhistamine was not altered in the rat cortex with ageing, whereas a significant decrease was seen in the hippocampus indicating an age-related loss in H_3 sensitivity.

The observed changes of H_3 receptor density in rat brain with age are probably not due to changes in the levels of histamine and an upregulation of H_3 receptors in the hippocampus might represent a compensatory mechanism for some loss in receptor

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functionality. The possible (pharmaco)therapeutic relevance of the age-related changes on H3-receptors in the hippocampus in relation to cognitive functions is discussed.

Introduction

Ageing has always both fascinated and terrified mankind. The interest in understanding and ultimately reversing the negative facets of ageing has been the incentive for increasing investment of human resources in pharmacotherapy to allow man to live longer and healthier.

The hippocampus is a structure of the brain known to play an important role in learning and memory formation. Lesions of this cerebral area cause remarkable impairment of cognitive functions, similar to those observed in senile dementia of the Alzheimer's type (SDAT) or in age-associated cognitive impairment (Smith *et al.*, 1988). Specifically, fibres of the enthorinal cortex represent a major input to the dentate gyrus molecular layer of the hippocampus (Cotman & Anderson, 1988). In fact, unilateral ablation of the enthorinal cortex caused 80% degeneration of the cholinergic projection to the molecular layer of the dentate gyrus (Cotman & Anderson, 1988). This observation suggests that this circuit is crucially involved in (patho)physiological changes in which cognitive functions are impaired, such as in Alzheimer's disease and, to a lesser extent, during the course of ageing. In general, age-related changes in neurotransmitters and receptors are restrained to certain brain areas in rodents and neurologically normal humans (Finch, 1993). A key point is that receptor affinity generally does not change (Finch, 1993). This implies that age changes at the level of synapses are due to altered relative amounts of particular proteins, rather than to the ichnographic "fundamental molecular ageing". The cholinergic system is one of the hippocampal neurotransmitter system best characterised both in physiological and pathological ageing (for review see Amenta *et al.*, 1991)). Age-related changes in the status of muscarinic cholinergic receptors have been well documented in both rodents and human brain (for review see Decker, 1987)). Consequently, possible strategies for the treatment of age-related cognitive impairment include the development of muscarinic cholinergic agonists (Roberts & Lazareno, 1989), or other agents responsible for increasing muscarinic transmission.

The involvement of histamine in memory-linked events has long been suggested (De Almeida & Izquierdo, 1986) but only over the recent years has substantial experimental evidence been gained in rodents (Cacabelos, 1996; Fernandeznovoa *et al.*, 1994; Meguro *et al.*, 1995; Miyazaki *et al.*, 1995; Miyazaki *et al.*, 1995; Vorobjev *et al.*, 1993). Moreover, using immunohistochemical techniques Airksinen *et al.* (1991) showed a significant decrease in histamine immunoreactivity in hypothalamic areas in which neurofibrillary tangles were found in Alzheimer patients.

The presynaptic histamine H3 autoreceptor is an important regulatory unit for histamine homeostasis in the CNS (Arrang *et al.*, 1983; Arrang *et al.*, 1987; Van der Werf *et al.*, 1987). The histamine H3 receptor not only regulates the release of histamine from histaminergic nerve terminals, but is also present in non-histaminergic nerve terminals, where it acts as a heteroreceptor modulating the release of other biogenic amines such as acetylcholine (Arrang *et al.*, 1995; Blandina *et al.*, 1996; Clapham & Kilpatrick, 1992), serotonine, noradrenaline, and dopamine in the CNS (Alves Rodrigues *et al.*, 1995; Leurs *et al.*, 1996; Schlicker *et al.*, 1988; Schlicker *et al.*, 1993; Schlicker *et al.*, 1989; Schlicker *et al.*, 1994). The reported H3 receptor modulation of the release of acetylcholine in the rat enthorinal cortex (Arrang *et al.*, 1995; Clapham & Kilpatrick, 1992) and the observation that histamine facilitates long term potentiation (LTP) induction and maintenance (Haas *et al.*, 1995), strongly support the hypothesis of the involvement of the histaminergic system, and the H3 receptor in particular, in hippocampal memory formation. Moreover, using behavioural studies in rodents, several authors have reported on the cognitive enhancing properties of the H3 receptor antagonist thioperamide, both under normal physiological conditions (Barnes *et al.*, 1993; Blandina *et al.*, 1996; Miyazaki *et al.*, 1995) and after scopolamine-induced learning deficits (Shichi *et al.*, 1995) or accelerated senescence (Meguro *et al.*, 1995). The presence of histamine H3 receptors in the hippocampus of the rodent (Cumming *et al.*, 1991; Jansen *et al.*, 1994; Pollard *et al.*, 1993; Yanai *et al.*, 1994) and of the primate (Martinez-Mir *et al.*, 1990) has previously been demonstrated using autoradiographic techniques. A pharmacological characterisation of the hippocampal H3 receptor has been described in detail for the first time in this dissertation (see chapter 5).

The present study was designed to investigate the influence of age on the density and physiology of histamine H3-receptor, as well as the levels of histamine in several brain regions. This study was performed using the first generation (F1) of hybrids rats (F344 x Lewis Brown Norway). These rats are of particular utility in gerontopharmacology as they in general seem to be less prone to develop age-associated pathologies, but still show clear age-related impairments of learning and memory (Van der Staay & Blokland, 1996).

Methods

Preparation of rat brain membranes

Male F1 (F344xLBN) rats (Harlan C.P.B., Zeist, The Netherlands) were decapitated and the brains were rapidly removed. Four independent batches of old rats (27-29 months old) membranes were prepared using n≥10 animals per batch, and three distinc batches of young (2 months of age) rats (10 animals/batch) membranes were prepared.

For each batch, several brain areas were dissected and hömogenised in 10 volumes (v:w) of ice-cold 50 mM Tris-HCl buffer (with 5 mM $MgCl_2$, 145 mM NaCl, pH 7.4 at 4°C) using a Ultra-Turrax blender and a Potter-Elvhjem homogeniser. This homogenate was centrifuged at 800 g for 10 min. The pellet was discarded and the supernatant centrifuged at 40,000 g during 40 min. The resulting pellet was rinsed twice under the same conditions. The final pellet was resuspended in 1.5 volumes (v:w) of the above described Tris-HCl buffer and stored at -80°C until the day of the experiment when it was diluted 2.5 (v:w) times in the same solution.

[^{125}I]-iodophenpropit binding assays

[^{125}I]-iodophenpropit binding assays were carried out according to the procedure described by Jansen *et al.* (1994). Saturation binding experiments of [^{125}I]-iodophenpropit (1950 Ci/mmol) were performed at 37 °C in 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 145 mM NaCl (pH 7.4 at 37°C) in a total incubation volume of 0.25 ml using polyethylene tubes. Determinations were performed in triplicate. Compounds were diluted in buffer. Rat brain membranes, previously prepared and kept at -80° C, were incubated for 60 min to reach equilibrium. Membranes were incubated with [^{125}I]-iodophenpropit in final concentrations ranging from 0.075 nM to 3 nM. In these assays, the non-specific binding was defined using 1 µM of (R)–α–methylhistamine. Incubations were started upon addition of 50 µl membranes (50-80 µg of protein per tube) and were terminated by the addition of 3 ml of ice-cold Tris-HCl buffer (pH 7.4, at 4°C), immediately followed by filtration through Whatman GF/C filters using a Brandel filtration apparatus. Filters were pretreated for at least 2 hours with 0.3% polyetheleneimine, reducing filter binding to less than 1% of the total radioactivity added. After filtration of the membranes the filters were washed twice with 3 ml of ice-cold Tris-HCl buffer. The amount of radioactivity bound to the membranes was not reduced by repetition of the washing procedure. The radioactivity bound to the filters was measured by an LKB gamma counter.

Protein concentrations were determined using the Bio-Rad Protein Assay kit (based on Bradford, 1976). Bovine serum albumin was used as standard.

HPLC determination of brain histamine content

10 Young (2 months old) and 10 old (27, 28 months old) male F1 rats were decapitated and nine (hypothalamus, striatum, cerebellum, cortex, forebrain, hippocampus, medulla, thalamus, pons) distinct brain regions were isolated quickly, blotted with filter paper, weighed and promptly homogenised in 10 volumes of 2% perchloric acid by sonication. Subsequently, the samples were centrifuged at 10,000 g

for 30 minutes at 4°C and the clear supernatant was injected directly onto an HPLC column. The chromatographic apparatus and conditions are described in detailed by Yamatodani *et al.* (1985). Histamine levels were expressed as nanomoles per gram of wet tissue.

Functional assays

Based on a combination of the methods described by Schlicker *et al.* (1989) and Van der Werf *et al.* (1987) slices of the rat cortex and hippocampus (0.3mm x 0.3mm) were incubated for 30 min in a Krebs-Ringer buffer (KRB) (in mM: NaCl 118, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.4, $NaHCO_3$ 25, glucose 10, pH 7.4 at 37° C when gassed with O_2/CO_2 (95%/5%)) containing 5 µCi [^{3}H]-noradrenaline (specific activity=38 Ci/mmol, NEN). After washing 4 times with 10 ml of KRB, the slices were preperfused with KRB (containing 1 µM desipramine and 1 µM phentolamine), for 60 min at a flow of 0.3 ml/min under constant gassing (95% O_2, 5% CO_2). Subsequently, seven 10 min fractions were collected, tritium overflow was evoked by a 200 seconds long electrical stimulation (rectangular pulses of 20 mA, 1ms, 0.3 Hz) during the second fraction (S_1) and the fifth fraction (S_2). RAMH was added right after the first stimulation (S_1). Finally, the slices were perfused for 20 min with 0.1 N HCl to determine the total content of tritium.

Data analysis

Saturation and binding experiments were evaluated on a Macintosh computer using the non-linear curve fitting programme LIGAND (Munson & Rodbard, 1980). With the aid of this programme binding curves were, respectively, fitted (unweighted) to a one and two independent sites models. The improvement of the fit for each model with additional parameters was evaluated based on the 'extra sum of squares' principal (Draper & Smith, 1966), taking $p<0.05$ to be significant.

The stimulation-evoked tritium overflow from brain slices was calculated by substraction of the basal efflux from the total efflux during stimulation and the subsequent 13 minutes and was expressed as percentage of tissue tritium at the onset of stimulation. For quantification of the effect of H_3-agonist (R)-α-methylhistamine on the electrically evoked tritium overflow, the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1). Data obtained from functional assays were fitted to a sigmoidal curve using the program Kaleidagraph (Albelbeck Software).

Results were expressed as mean±SEM of the number of independent experiments indicated. Statistical differences among the two animal groups examined were assessed by a paired (for the binding experiments and histamine levels measurements) and

unpaired (for the functional experiments) Student *t*-test analysis, $p<0.05$ was taken as significant.

Chemicals

[^{125}I]-Iodophenpropit was labelled to a specific activity of 1950 Ci/mmol as described by Menge *et al,* (1992). [^{3}H]-Noradrenaline (specific activity=38 Ci/mmol) was purchased from NEN, Netherlands. (R)-α−methylhistamine maleate and polyethylenimine were purchased from Aldrich-Chemie. Perchloric acid and histamine diphosphate were obtained from Sigma, potassium dihydrogen phosphate, *o*-phthalaldehyde, sodium hydroxide and sulphuric acid of super-special grade were purchased from Baker. Other chemicals were of analytical-reagent grade and were used without further purification. Glass-distilled water purified further with a Milli-Q system was used throughout the HPLC separations.

Results

[^{125}I]-iodophenpropit binding assays

[^{125}I]-iodophenpropit bound specifically to membranes of the three regions of the rat brain studied. The binding parameters (B_{max} and K_d) obtained for the rat brain cortex, hippocampus and striatum are summarised on table 1. The K_d values for the binding of [^{125}I]-iodophenpropit were similar in all the cerebral regions investigated and did not change with age (table 1).

Table 1 Dissociation constants (K_D) and number of specific binding sites (B_{max}) for the binding of [^{125}I]-iodophenpropit to membranes of rat brain. The radioligand binding studies were performed as described in Methods. Data represent mean±SEM of four independent experiments performed in triplicate.

Brain area	K_D *(Young)* (nM)	B_{max} *(Young)* fmol/mg prot.	K_D *(Old)* (nM)	B_{max} *(Old)* fmol/mg prot.
Hippocampus	0.63±0.15	123±18	0.83±0.13	241±16
Cortex	0.63±0.12	173±18	0.57±0.10	109±64
Striatum	0.60±0.10	236±16	0.90±0.30	256±17

In the hippocampus, the maximal density of [^{125}I]-iodophenpropit binding sites drastically increased in aged animals (95%, $p<0.01$, Fig. 1 A) whereas a decrease of 35% ($p<0.01$) was observed in the cerebral cortex (Fig. 1B). No difference was detected in the density of [^{125}I]-iodophenpropit binding sites in the rat striatum ($p>0.5$, table 1). No significant age-related differences were detected in the total protein content (per mg of tissue) of the several brain regions investigated (data not shown).

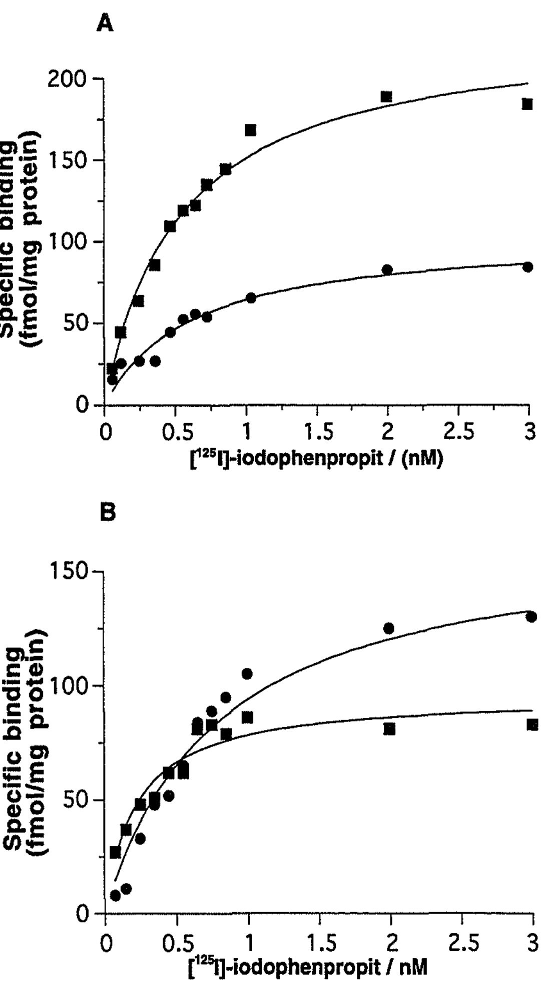

Figure 1 Saturation binding of [^{125}I]-iodophenpropit to membranes from young (filled squares) and old (filled circles) rat (**A**) hippocampus and (**B**) cerebral cortex. The nonspecific binding was defined in the presence of 1 μM (R)-α-methylhistamine. Results shown are from one representative experiment of four performed in triplicate. Student's *t*-test was used for statistical analysis.

Histamine levels

From the nine distinct brain regions studied for their histamine content only the hypothalamus showed a significant increase ($p<0.05$, 23%) in the total amount of histamine with age. All the other brain areas showed no age related change in their histamine levels ($p>0.05$; Fig.2).

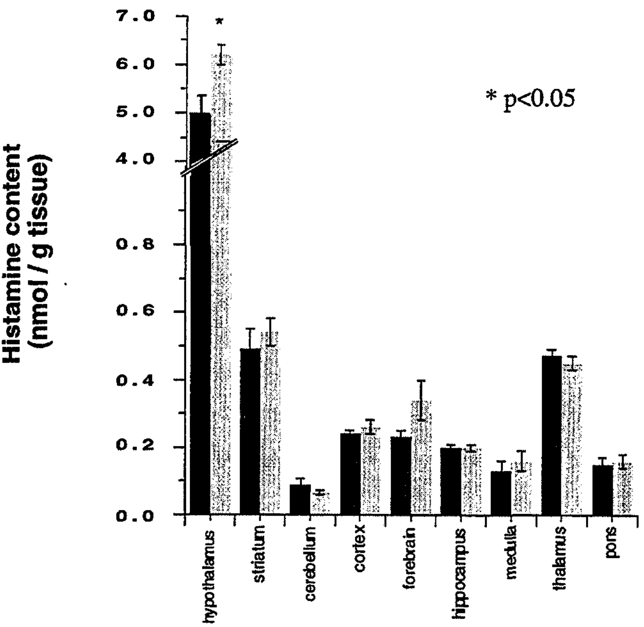

Figure 2 Regional brain histamine concentrations in young (black columns) and old (grey columns) rats. The data represent the mean and standard error of ten young rats and ten old rats. Student's *t*-test was used for statistical analysis.

Functional H_3 receptor assay

On the H_3-mediated inhibition of the electrically induced release of [^{3}H]-noradrenaline no changes were observed on the ratio S_2/S_1 in ageing. Both the levels of basal release and total content of [^{3}H]-noradrenaline remained unchanged with age and between the two brain areas (data not shown). In the cerebral cortex, no changes were detected on the $\%S_1$ values (data not shown), whereas in the hippocampus a significant age-related decrease was observed: for young rats $\%S_1$ values of 11.4±1.7 and for old rats 8.54±1.1 (n=7, $p<0.05$) were obtained. $\%S_2$ values showed the same tendecy yielding a value of 8.42±1.4 in young rats and 6.3±0.9 in old rats (n=7, $p<0.05$). The level of

maximum inhibition was obtained using 1 μM (R)-α-methylhistamine and an age-related decrease in those levels was observed in both the cerebral cortex: 49±0.7% versus 37±2.6% ($p<0.01$, n=6); and in the hippocampus: 47±1.1 % in young rats and 39±2.0 % (n=7) in old rats ($p<0.01$, n=7). No major changes were noticed in the pD_2 values for (R)-α-methylhistamine in the cerebral cortex: 7.6±0.1 in young rats versus 7.8±0.1 in old rats (Figure 3A). In the hippocampus, however, an age-related decrease in the pD_2 for (R)-α-methylhistamine was observed with ageing: 7.7±0.02 in the young rats versus 7.2±0.2 in the old rats ($p<0.05$; Figure 3 B).

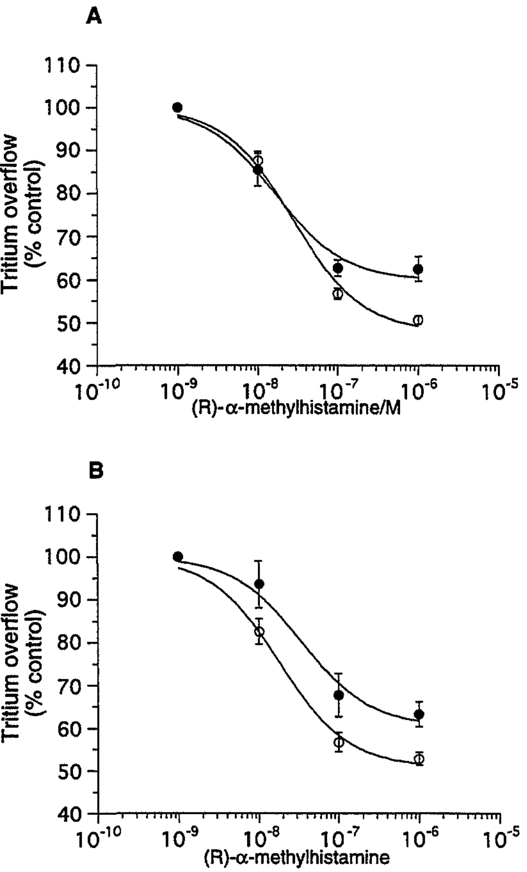

Figure 3 Concentration response curve of (R)-α-methylhistamine for the inhibition of the electrically stimulated tritium overflow from superfused rat brain slices preincubated with [^{3}H]-noradrenaline. Open circles represent data from young rats and filled circles from old rats; (A) in the cerebral cortex, (B) in the hippocampus. Each point represents mean±SEM of six or seven experiments performed in duplicate. Student's *t*-test was used for statistical analysis.

Discussion

Deterioration of cholinergic transmission in the enthorinal cortex is often linked to memory loss (Bierer *et al.*, 1995). This observation together with the reported H3 receptor modulation of the release of acetylcholine in the enthorinal cortex (Arrang *et al.*, 1995; Clapham & Kilpatrick, 1992) and in the cerebral cortex (Blandina *et al.*, 1996) supports the hypothesis of the involvement of the histaminergic system, and the H3 receptor in particular, in hippocampal memory formation. Following the characterisation of the hippocampal H3 receptor (chapter 5) and the recently reported behavioural studies in rodents on cognitive enhancing properties of the H3 receptor antagonist thioperamide (Barnes *et al.*, 1993; Meguro *et al.*, 1995; Miyazaki *et al.*, 1995; Shichi *et al.*, 1995) we investigated, in this section, possible age-related changes in the density of histamine H3 receptors and function in several brain areas.

Using the H3 receptor antagonist [^{125}I]-iodophenpropit, we performed saturation binding assays with membranes of different regions of the brain of young (2 months old) and old (27-29 months old) rats. From these assays we observed no significant difference in the dissociation constant of the binding of [^{125}I]-iodophenpropit between the brain regions or changes therein with age. This result confirms the observation of (Finch, 1993) that most age-related changes in neurotransmitters receptors do not involve their affinity for specific ligands.

Our findings show, on the other hand, important and large age-associated changes in the density of H3 receptors in some of the brain areas studied. No significant alterations in the number of H3 receptors was observed in the rat striatum, whereas a significant decrease of 35% in the H3-receptor density was seen in the cerebral cortex. Most surprisingly, rat hippocampus showed a dramatic, almost two fold, increase in the number of H3 receptors with ageing. The H3 receptor downregulation observed in the cerebral cortex and the unchanged density found in the striatum raise the idea of the involvement of a specific regulatory mechanism for the H3 receptor in different brain regions. Our data contrast with what is seen in the hippocampus for several other receptors including the histamine H_1 receptor (Yanai *et al.*, 1992) and the muscarinic receptors (Amenta *et al.*, 1995). Hippocampal M_1 receptors were reported to decrease with ageing whereas no changes in the density of M_2 receptors were detected (Amenta *et al.*, 1995). M_1 downregulation is in accordance with age-related cognitive impairments often associated with cholinergic transmission deficiencies (Bierer *et al.*, 1995).

So far *in vitro* functionality of the hippocampal H3 receptor has only been described on the inhibition of noradrenaline release (chapter 5). The changes observed on the functional H3 receptor assay showed an age-related decrease in the capacity in hippocampal neurons to respond to electrical stimulated release of [^{3}H]-noradrenaline,

whereas basal release and total levels of [^{3}H]-noradrenaline uptake remained unaltered. In the cortex, however, no significant changes were detected in either the stimulated or the basal and total content of [^{3}H]-noradrenaline. Furthermore, an important decrease of H3 receptor functionality in the hippocampus was detected whereas minor changes were observed in the cerebral cortex of old rats. This is the first report on decrease of H3 receptor function with age. Previously (Schlicker *et al.*, 1991) observed no impairment of H3 receptor inhibition of serotonin release in the rat cortex.

Based on previous studies on the cognitive enhancing properties of the α_2 agonist clonidine (Arnsten & Goldman-Rakic, 1984; Sara *et al.*, 1987), Sara (1989) published an interesting report on noradrenergic-cholinergic interaction and its possible role in senile dementia associated memory disfunction (Sara, 1989). Using cognitive behavioural studies in rats which hippocampal cholinergic activity was reduced by specific neuronal lesions, they showed that lesioned rats treated with clonidine had a performance indistinguishable from the non-lesioned rats while rats which had suffered cholinergic damage performed poorly. This observation supported the author's hypothesis that when there is a reduction in the cholinergic function, a corresponding decrease in noradrenaline release (by clonidine) reduces the noradrenergic inhibitory influence on acetylcholine release in terminals of spared cholinergic neurons. Furthermore, this explains the previous experimental observation by Arnsten *et al.* (1984) that clonidine only affected the performance of monkeys in which the cholinergic system was impaired either by lesions or by ageing (Arnsten & Goldman-Rakic, 1984) and had no effect on young undamaged brains. If a fine tuning between noradrenergic and cholinergic transmission is important to assure cognitive performance in senile brains, decreases in the H3 receptor mediated inhibition of the noradrenergic system in the hippocampus like the ones observed in our study can account for important noradrenaline mediated cholinergic inhibition, and consequently cognitive dysfunction. Such a mechanism can, however, not explain the observed cognitive enhancing properties of H3 receptor antagonists. On the other hand, as no changes were detected in the histamine levels of aged rats, direct inhibition by histamine, acting on the H3 receptor present on cholinergic nerve terminals, of the cholinergic system could also play an important role. This mechanism would explain the described cognitive enhancing properties of the H3 receptor antagonist thioperamide and clobenpropit under conditions in which cognitive functions are impaired (Blandina *et al.*, 1996; Shichi *et al.*, 1995). This hypothesis could be of relevance for the development of non-toxic, brain penetrating H3 receptor antagonists (e.g. as the ones described in chapter 3) as potential ameliorating agents in conditions in which cognitive functions are affected such as Alzheimer's disease, SDAT, or attention deficit disorder. Functional studies on the H3-modulation of hippocampal release of acetylcholine in old and young rats would be of great interest and are currently under investigation. These

studies might provide the mechanistic basis of the observed cognitive enhancing properties of H3 receptor antagonist

Taken together, these data indicate that a drastic H3-receptor upregulation in the hippocampus and the moderate cortical downregulation in old rats are unlikely to be consequences of changes in the histamine levels in those parts of the brain but might be the result of a re-adaptive mechanism of the brain to the age-associated changes in the functionality of the H3 receptor. Namely, in the hippocampus a pronounced decrease in neuronal response to electrical stimulus as well as a decrease in H3 receptor functionality were observed whereas in the cortex only the H3 receptor functionality seemed to be slightly altered in ageing. Taking into account that in the hippocampus the average number of H3 receptors is almost doubled in aged rats and yet significant impairments are observed in their functional properties, further support is given for the involvement of the H3 receptor in age (or pathology) associated decreases in cognitive performances.

Although this study on the age-dependent changes of the histamine H3 receptor is preliminary, some light has been shed on the involvement of the H3 receptor in age-related impairment of cognitive functions.

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Chapter 7

SUMMARY AND FINAL DISCUSSION

Neuronal histamine is involved in several physiological conditions, such as arousal, locomotor activity, food intake, and learning and memory processes. H3 receptor antagonists enhance neuronal histamine release and, therefore, activate via stimulation of post synaptic histamine H_1 and H_2 receptors, the histaminergic neuronal system. The opposite effects are accomplished by administration of histamine H3 agonists. Equally interesting was the discovery of histamine H3 heteroreceptors that play a similar role in the release of other neurotransmitters. Histamine H3 heteroreceptors have been identified on various monoaminergic nerve terminals, such as the noradrenergic, dopaminergic, serotonergic, and cholinergic.

A comprehensive description of the neuronal histaminergic system and, in particular, of the pharmacological and (patho)physiological roles of the histamine H3 receptor in the CNS is given in **chapter 1**. In this chapter, potential applications of H3 receptor ligands as therapeutical agents are discussed in relation to both their modulatory effect on the release of neurotransmitters and the (neuro)pathologies associated with each of the neurotransmitters discussed. Emphasis is put on recent evidence obtained by several research groups on the involvement of histamine in memory formation.

Clozapine as a drug acting on the H3 receptor

In view of recent studies suggesting the involvement of histamine with mental disorders such as schizophrenia, Parkinson's Disease and Alzheimer's Disease we investigated in **chapter 2** the affinity of clozapine and other neuroleptics for the H3 receptor. Using the radiolabelled H3 antagonist [^{125}I]-iodophenpropit in competition binding assays and H3 modulation of the release of serotonin, we observed an interaction of clozapine with the H3 receptor in the high nanomolar range and an antagonistic effect on the H3 receptor. Other neuroleptics tested showed much lower affinities for the H3 receptor. We conclude that if the K_i value for clozapine at the human H3 receptor is similar to the value obtained for its rat counterpart, brain clozapine concentrations might be high enough for a functional interaction with the H3 receptor under clinical conditions. From the low H3-receptor affinity displayed by the two major clozapine metabolites (clozapine-N-oxide and N-desmethylclozapine) it can also be concluded that the two metabolites will not contribute to the H3 receptor antagonism in the clinical profile of clozapine.

Qualitative SAR for binding of clozapine and immepip analogues to the H_3 receptor

As no structural information is yet available for the H_3 receptor an approach based on comparison of affinities/activities of structurally related compounds is the only way to study its ligand-binding characteristics. In view of the unexpectedly high affinity for the H_3 receptor observed for clozapine, we tested (**chapter 2**) the affinity of several clozapine metabolites and analogues for the histamine H_3 receptor. Qualitative structure affinity relationships were derived for the tested compounds. In the clozapine molecule four structurally different moieties were considered. In comparison with the affinity for the H_3 receptor shown by clozapine, the following main conclusions can be drawn: the 4-piperazinyl region does not allow substituents longer than a CH_3 or electronegative atoms such as an O; substitutions at the 5-diazepine position do not drastically alter the affinity for the H_3 receptor, although a basic nitrogen is favoured over CH_2, O, or S; the 8 position in phenyl ring I is an important modulatory site for H_3 affinity, electronegative substituents such as chloro and fluoro in this aromatic group increase the affinity. When these substituents are present at the phenyl group II, these substituents totally disable binding to the H_3 receptor.

From an extensive characterisation of the pharmacology of the H_3 receptor agonist, immepip, and its short (VUF 4735) and long (VUF 4929) analogues (**chapter 3**) we concluded that when the amino group is part of a piperidine ring, a 4-carbon chain between the imidazole ring of the molecule of histamine and the amino function seem to be the ideal for H_3 agonism. Extending or shortening this distance leads to complete loss of agonistic activity.

H_3 receptor subtypes

Following a previous report by our research group on discriminative properties of a series of aliphatic histamine homologues, we studied, in **chapter 3** immepip and its lower (VUF 4735) and higher (VUF 4929) homologues as ligands for the histamine H_3 receptor in two binding assays (using $[^{125}I]$-iodophenpropit and $[^{3}H]$-N^{α}-methylhistamine, as radiolabelled ligands) in rat cerebral cortex membranes, and two functional H_3 receptor models (inhibition of the neurogenic contraction in the guinea-pig jejunum and inhibition of $[^{3}H]$-noradrenaline release in rat cerebral cortex slices).

Immepip showed high affinity for the binding of $[^{3}H]$-N^{α}-methylhistamine (pK_i=8.7) which was comparable to the high affinity component of the binding of $[^{125}I]$-iodophenpropit ($pK_{i(high)}$=8.5). VUF 4735 also showed similar affinities in both binding assays (pK_i=6.1). The binding data obtained for VUF 4929, however, showed major discrepancies between the two assays.

Functionally, the immepip homologues acted as competitive H_3-receptor antagonists in both systems. The potencies (pA_2 values) observed at the guinea pig jejunum were 8.4

and 6.2 for VUF 4929 and VUF 4735, respectively, whereas on the electrically evoked release of [^{3}H]-noradrenaline from cortical slices the pA_2 values were 7.1 and 5.5 for VUF 4929 and VUF 4735, respectively. Moreover, the H_3-receptor agonist immepip, but not the H_3-agonist (R)–α–methylhistamine, showed almost a 10-fold higher agonistic potency in the rat cerebral cortex than in the guinea-pig jejunum.
We concluded from this study that immepip homologues revealed important differences in different H_3 receptor assays; such differences would not be expected to occur if the same receptor subtype was present in the tissues studied. With this study we have given further support to the existence of histamine H_3-receptor subtypes for which several ligands show clear discriminative behaviour. Due to the growing interest in H_3 ligands as putative therapeutic agents in several CNS disorders, detailed information with respect to H_3-receptor heterogeneity is mandatory. Moreover, immepip and its ligands might become attractive compounds for drug development as they do not possess the toxic (iso)thiourea moieties of thioperamide and clobenpropit and because they seem to be able to discriminate between central and peripheral H_3-receptors.

Selectivity of H3 receptor radiolabelled antagonists
In **chapter 4** we characterise the binding of the histamine H_3 receptor antagonist [^{3}H]-thioperamide to rat cerebral cortical membranes. From this study we could draw important conclusions regarding the selectivity of the binding of H_3 receptor agonists and antagonists. Summarising, [^{3}H]-thioperamide bound to rat brain cortical membranes in a saturable and reversible manner showing high and low affinity components. The high affinity site is likely to represent the histamine H_3 receptor as the binding is displaced by (R) and (S)-α–methylhistamine in a stereoselective manner and by several other H_3 ligands with a pharmacological profile of the H_3 receptor. At nanomolar concentrations binding of [^{3}H]-thioperamide to a low affinity non-H_3 receptor binding site(s) increases steeply, reaching, at saturation, a density 30 fold higher than the number of H_3 receptors. Also other H_3 antagonists we tested show high affinity for the non-H_3 receptor sites. Since thioperamide was previously reported to bind to cytochrome P_{450} enzymes and this enzymatic complex is present in relatively high amounts in the brain cortex the binding to cytochrome P_{450} isoenzymes may explain the high density of the binding observed at higher concentrations of [^{3}H]-thioperamide. In fact, three non-selective P450 inhibitors, metyrapon, SKF 525 A, and imidazole competitively inhibited the binding of nanomolar concentrations of [^{3}H]-thioperamide to rat cortical membranes. Moreover, similar results were obtained with the P_{450} inhibitors when rat liver microssomes (an organ not known to contain histamine H_3 receptors) were used in [^{3}H]-thioperamide binding assays. Therefore, based on this study, we state that it would be judicious to use H_3 agonists rather than H_3 antagonists to delineate specific (and non-specific) binding of radioactively labelled

H3 antagonists. We also conclude in **chapter 4** that from the four H3 receptor antagonists used to the displace the [^{3}H]-thioperamide binding, iodophenpropit was the one which best discriminated between H3 and non-H3 binding sites.

This results was further confirmed when, in **chapter 5**, we characterised the histamine H3 receptor in the rat brain hippocampus using the H3 receptor antagonist [^{125}I]-iodophenpropit. Up to a concentration of 1 μM, the H3 receptor agonist (R)-α-methylhistamine displaced 30% of the total binding of [^{125}I]-iodophenpropit whereas H3-antagonists displaced 50-60% of the bound [^{125}I]-iodophenpropit, giving further evidence for the binding of H3-receptor antagonists to a non-H3 receptor site(s). This was not so obvious in the rat cerebral cortex in other studies using the same radioligand, and taking into account a higher abundance of P450 isoenzymes in the hippocampus in relation to the cortex, we strongly suspect this other secondary non-H3 receptor site(s) to be cytochrome P450 isoenzymes. We again emphasise that due attention should be given to these observations in studies for which receptor densities are of relevant importance.

In an attempt, previous to the studies above mentioned, to characterise the histamine H3 receptor in human brains of healthy and schizophrenic patients (A. Alves-Rodrigues, unpublished data) we could displace the binding of 0.25 nM [^{125}I]-iodophenpropit by H3 antagonists such as thioperamide but barely any binding could be displaced by the H3 receptor agonist (R)-α-methylhistamine (Figure 1).

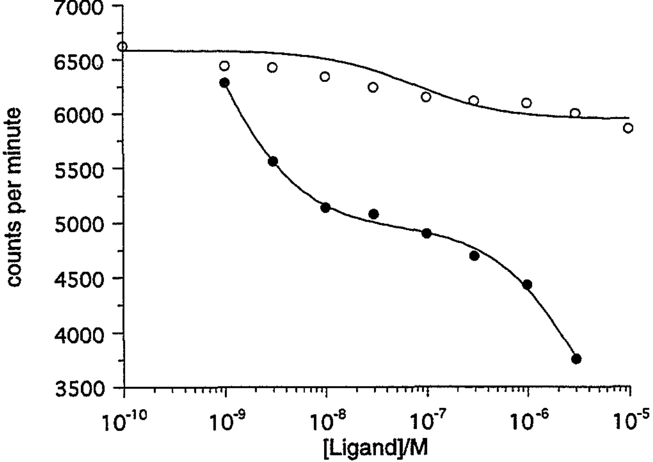


Figure 1 Competition by R-α-methylhistamine (open circles) and thioperamide (filled circles) for the binding of 0.25 nM [^{125}I]-iodophenpropit to membranes of human brain. Data are from one representative experiment out of 6 performed in triplicate.

In light of what we describe in **chapters 4** and **5** it is likely that a much higher density of P450 isoenzymes compared to the number of H3 receptors in the human brain is the cause of the absence of displacement of [^{125}I]-iodophenpropit by H3 receptor agonists. It can be seen, as well, that a rather shallow thioperamide displacement curve is obtained in human tissue, indicating binding to more than one population of sites similar to what happens with [^{3}H]-thioperamide in the rat cerebral cortex. To extend this study, competition binding assays in the presence of a non-selective P450 isoenzyme inhibitor could elucidate whether the dense non-H3 component of the binding of H3 antagonists to human brain membranes are indeed P450 isoenzymes and, this way, allow the use of radiolabelled H3 receptor antagonists in binding studies where P450 isoenzymes are in abundance in relation to H3 receptors. Preliminary studies showed that clozapine, a non-imidazole compound, was the only H3 receptor antagonist tested (out of 8 different compounds) which failed to displace any of the binding of [^{125}I]-iodophenpropit.

The histamine H3 receptor in the hippocampus. Possible involvement in hippocampal memory formation

The characterisation of the histamine H3 receptor in the rat hippocampus in **chapter 5** revealed labelling of a single class of binding sites with a density lower than the one previously reported for the rat cerebral cortex. [^{125}I]-iodophenpropit binding to rat hippocampal membranes was readily displaced by histamine H3 receptor agonists and antagonists, with K_i values within the range expected for binding to the histamine H3 receptor and comparable to the values obtained for the H3 receptor present in the rat brain cortex. Stereoselectivity between the (R) and (S) enantiomers of the H3 receptor agonist α-methylhistamine was observed. In the presence of GTPγS a rightwards shift occurred for the high affinity site of the binding of (R)-α-methylhistamine. In **chapter 5** we also showed the functionality of the H3 receptor in the rat hippocampus; electrically stimulated [^{3}H]-noradrenaline release from hippocampal slices could be effectively reduced by H3 receptor stimulation. No significant differences were detected between the levels of maximum inhibition of the electrically evoked release of [^{3}H]-noradrenaline or with the control ratio S_2/S_1 between the hippocampus and the cerebral cortex. Both the pD_2 values obtained for (R) and (S)-α-methylhistamine, and the pA_2 values for thioperamide and burimamide showed similar values in the two cerebral areas studied. In **chapter 5** we delineated conditions to study the histamine H3 receptor in the hippocampus and the potential changes in its pharmacological and/or physiological properties.

Following the characterisation of the H3 receptor in the rat hippocampus we then proceeded in **chapter 6** with the study of age-related changes in the density and function of the histamine H3 receptor in the rat hippocampus. Additionally, using HPLC techniques, we measured the levels of histamine in nine different regions of the rat brain. An H3 receptor upregulation of 95% was observed in the hippocampus whereas the

cerebral cortex revealed a decrease of 35% in the number of H3 receptors and no age-related changes were detected in the striatum. Histamine levels showed an increase of 23% in the hypothalamus and no significant changes in the other brain areas studied including the hippocampus, cortex and striatum. In functional studies on histamine H3 receptor inhibition of the electrically evoked release of $[^3H]$-noradrenaline, a lower stimulation (S_1 and S_2) was obtained in the hippocampus whereas no changes occured in the rat cortex. Both the hippocampus and the cortex showed significant age-related decreases in the maximal level of inhibition. The pD_2 value of (R)-α-methylhistamine showed no change in the rat cortex with ageing, whereas a significant decrease was seen in the hippocampus indicating an age-related loss in H3 sensitivity.
Taken together, **chapter 6** indicates that a drastic H3-receptor upregulation in the hippocampus and a moderate cortical downregulation in old rats iss not due to changes in the histamine levels in those parts of the brain but might be the result of a re-adaptive mechanism of the brain to the age-associated changes in the functionality of the H3 receptor. One can speculate that, in the hippocampus, an increase in the number of receptors can, to some extend, compensate for a less efficient H3 receptor in aged rats. In both brain areas a decrease on the maximal inhibition produced by the H3 agonist (R)-α-methylhistamine was seen in ageing.

The studies described in this dissertation have contributed to a better understanding of the pharmacology and physiological changes the histamine H3 receptor. Development of H3 receptor ligands that do not bind with high affinity to the non-H3 receptor site(s) detected by current H3 receptor antagonists could be of most utility. Good candidates for these ligands are non-imidazole compounds such as the ones described in chapter 2, although higher affinities are prefered to improve receptor selectivity. The existence of H3 receptor subtypes and ligands that show discriminative affinities for the central and peripheral H3 receptors, like the immepip analogues studied in chapter 3, would be of interest in therapy. Drug design of H3 ligands that penetrate the blood brain barrier has shown several difficulties, probably due to metabolism of the compounds in organs in which cytochrome P450 enzymes exist in high densities. Finally, better H3 receptor ligands, as drugs or as radiolabelled compounds for brain imaging studies such as PET, would certainly help to further elucidate the rather promising role of the histamine H3 receptor in brain functions such as learning and memory.

Samenvatting en afsluitende discussie

Behorende bij het proefschrift "De histamine H3 receptor in de hersenen van de rat. Farmacologische en (patho)fysiologische aspecten"
(Vertaald door Sylvia Lemstra en Ingrid van den Nieuwenhof)

In het centrale zenuwstelsel is histamine betrokken bij diverse fysiologische processen, zoals opwinding, locomotie, voedselopname, leer- en geheugenprocessen. H3 receptor antagonisten bevorderen de neuronale histamineafgifte en op deze manier activeren ze, via stimulatie van postsynaptische histamine H1 en H2 receptoren, het histaminerge neuronale systeem. De tegenovergestelde effecten worden veroorzaakt door histamine H3 receptor agonisten. Van groot belang was de ontdekking dat histamine H3 heteroreceptoren ook de afgifte van andere neurotransmitters reguleren. Deze histamine H3 heteroreceptoren zijn ondekt op de uiteinden van verschillende monoaminerge zenuwen, zoals de noradrenerge, dopaminerge, serotonerge, and cholinerge zenuwen. Een algemene beschrijving van het neuronale histaminerge systeem en in het bijzonder van de farmacologische en (patho)fysiologische rol van de histamine H3 receptor in het centraal zenuwstelsel is beschreven in **hoofdstuk 1**. In dit hoofdstuk worden bovendien mogelijke therapeutische toepassingen van H3 receptor liganden beschreven. Hierbij wordt getracht een relatie met het modulerend effect op de afgifte van de verschillende neurotransmitters te vinden. Speciale aandacht is besteed aan recent verkregen bewijzen omtrent de betrokkenheid van histamine in geheugenvorming.

Clozapine als een geneesmiddel werkend op de H3 receptor

Op basis van recente studies, die de betrokkenheid van histamine bij schizophrenie, de ziekte van Parkinson en de ziekte van Alzheimer doen vermoeden, hebben wij in **hoofdstuk 2** de affiniteit van clozapine en andere neuroleptica voor de H3 receptor onderzocht. Gebruik makend van de radioactief gelabelde H3 antagonist [^{125}I]-iodophenpropit en de H3 receptor gemedieerde afgifte van serotonine uit hersenslices, werd een interactie van clozapine met de H3 receptor in nanomolair concentraties en een antagonistisch effect op de H3 receptor waargenomen. Andere geteste neuroleptica vertoonden een veel lagere affiniteit voor de H3 receptor. Wij concluderen dat als de affiniteit van clozapine voor de humane H3 receptor gelijk is aan de waarde verkregen voor de H3 receptor van de rat, de clozapine concentratie in de hersenen mogelijk hoog genoeg is voor een functionele interactie met de H3 receptor onder klinische condities.

Door de lage H3 receptor affiniteit van de twee belangrijkste clozapine metabolieten (clozapine-N-oxide en N-desmethylclozapine) kan er geconcludeerd worden dat de twee metabolieten niet verantwoordelijk zijn voor een mogelijke bijdrage van H3 receptor antagonisme tot het klinische profiel van clozapine.

Kwalitatieve SAR voor binding van clozapine en immepip analoga aan de H3 receptor

Omdat er nog geen informatie beschikbaar is over de H3 receptor structuur is een vergelijking van affiniteiten/activiteiten van structureel verwante verbindingen de enige manier om de ligand-bindingskarakteristieken te bestuderen. Op basis van de onverwacht hoge affiniteit van clozapine voor de H3 receptor werd de affiniteit van diverse clozapine metabolieten en analoga voor de histamine H3 receptor getest (**hoofdstuk 2**). Kwalitatieve structuur-affiniteit-relatie's werden afgeleid voor de geteste verbindingen. In het clozapine molecuul werden vier verschillende structuurelementen onderscheiden. Voor een goede H3 receptor affiniteit dienen clozapine-analoga aan de volgende eisen te voldoen: de 4-piperazinyl ring staat geen substituenten toe die groter zijn dan een CH_3 noch electronegatieve atomen zoals een O; substituties op de 5-diazepine positie veranderen de affiniteit voor de H3 receptor niet drastisch, hoewel een basische stikstof de voorkeur heeft ten opzichte van CH_2, O, of S; de 8 positie in fenyl ring I is een belangrijke modulatie plaats voor H3 affiniteit, electronegatieve substituenten als chloor en fluor geven een toename van de affiniteit. Als deze substituenten aanwezig zijn op de fenyl groep II, zijn de verbindingen niet in staat te binden aan de H3 receptor.

Na een uitgebreide farmacologische karakterisering van de H3 receptor agonist, immepip, de "verkorte" (VUF 4735) en de "verlengde" (VUF 4929) immepip analoga (**hoofdstuk 3**), konden we concluderen dat, wanneer de amino groep onderdeel is van een piperidine ring, 4 koolstof atomen tussen de imidazol-ring en de amino functie optimaal is voor H3 agonisme. Verlenging of inkorting van deze afstand leid tot compleet verlies van de agonistische activiteit.

H3 receptor subtypes

Aan de hand van een eerder verschenen artikel van onze onderzoeksgroep over een serie alifatische histamine homologen, onderzochten we, in **hoofdstuk 3** immepip en zijn lagere (VUF 4735) en hogere (VUF 4929) homologen als liganden voor de histamine H3 receptor in twee bindingsassays ([^{125}I]-iodophenpropit en [^{3}H]-N$^{\alpha}$-methylhistamine) en in twee functionele H3 receptor modellen (remming van de neurogene contractie van het jejunum van de cavia en de remming van [^{3}H]-noradrenaline afgifte in slices van de cerebrale cortex van de rat).

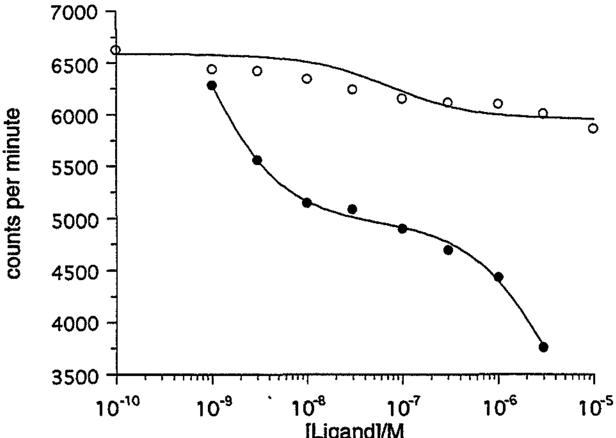
Immepip vertoonde hoge affiniteit voor de binding van [^{3}H]-N$^\alpha$-methylhistamine (pK_i=8.7) wat vergelijkbaar was met de hoge affiniteitscomponent voor de binding van [^{125}I]-iodophenpropit (pK_i(high)=8.5). VUF 4735 gaf vergelijkbare affiniteiten in beide assays (pK_i=6.1). De verkregen waarden voor VUF 4929 vertoonden echter grote verschillen tussen de twee assays.
Functioneel gedroegen de immepip homologen zich als competitieve H_3 receptor antagonisten in beide systemen. De potenties (pA_2 waarden) gemeten in het jejunum van de cavia waren 8.4 en 6.2 voor VUF 4929 en VUF 4735, respectievelijk, terwijl in de hersenen van de rat de pA_2 waarden 7.1 and 5.5 waren, voor VUF 4929 en VUF 4735 respectievelijk. In tegenstelling tot de H_3 agonist (R)-α-methylhistamine, was de H_3 receptor agonist immepip, bijna 10 maal potenter in de cerebrale cortex van de rat, dan in het jejunum van de cavia. We concluderen op basis van deze studie dat immepip homologen belangrijke verschillen onthullen in beide H_3 receptor assays; deze verschillen zou men niet verwachten als hetzelfde receptor subtype aanwezig is in de bestudeerde weefsels. Met deze studie hebben we opnieuw aanwijzingen gevonden dat er histamine H_3 receptor subtypes bestaan. Als gevolg van de groeiende interesse in H_3 liganden voor therapeutische toepassingen in diverse stoornissen van het centrale zenuwstelsel, is verdere informatie met betrekking tot de heterogeniteit van de H_3 receptor noodzakelijk. Immepip en zijn analoga zouden mogelijk goede verbindingen zijn voor de ontwikkeling van geneesmiddelen; deze verbindingen bevatten geen toxische (iso)thioureumgroep, zoals thioperamide en clobenpropit. Bovendien lijken deze stoffen onderscheid te maken tussen centrale en perifere H_3-receptoren.

Selectiviteit van radioactief gelabelde H_3 receptor antagonisten

In **hoofdstuk 4** wordt de binding van de histamine H_3 receptor antagonist [^{3}H]-thioperamide aan de membranen van de cerebrale cortex van de rat beschreven. Op basis van deze studie konden belangrijke conclusies getrokken worden met betrekking tot de selectiviteit van de binding van H_3 receptor agonisten en antagonisten. [^{3}H]-thioperamide bind aan cerebrale cortex membranen op een verzadigbare en reversibele manier aan twee bindingsplaatsen. De hoge affiniteit bindingsplaats is hoogstwaarschijnlijk de histamine H_3 receptor. De binding aan deze bindingsplaats wordt stereoselectief verdrongen door (R) en (S)-α-methylhistamine en door verschillende andere H_3 liganden met het farmacologisch profiel van de H_3 receptor. In nanomolair concentraties bind [^{3}H]-thioperamide ook aan een lage affiniteits, niet-H_3 receptor-bindingsplaats. De dichtheid van deze bindingsplaats is 30 keer hoger dan de H_3 receptordichtheid.
Ook andere geteste H_3 antagonisten vertoonden een redelijke affiniteit voor de niet-H_3 receptor bindingsplaatsen. Omdat al eerder gepubliceerd was dat thioperamide kan

binden aan cytochroom P450 en dit enzymatische complex ook in relatief hoge hoeveelheden aanwezig is in de hersenen, kan de binding aan cytochroom P450 isoenzymen de hoge dichtheid van de lage affiniteits [^{3}H]-thioperamide bindingsplaats verklaren. Drie aselectieve cytochroom P450 remmers, metyrapon, SKF 525A en imidazol, remden ook de binding van nanomolair concentraties [^{3}H]-thioperamide. Vergelijkbare resultaten werden verkregen met de cytochroom P450 remmers in [^{3}H]-thioperamide bindingsexperimenten in lever microsomen van de rat (een orgaan dat, zover bekend, geen histamine H3 receptoren bevat). Op basis van deze resultaten, stellen we voor dat het verstandig is om H3 agonisten in plaats van H3 antagonisten te gebruiken om de specifieke (en niet specifieke) binding van radioactief gelabelde H3 antagonisten te beschrijven. Ook werd er in **hoofdstuk 4** geconcludeerd dat van de vier gebruikte H3 receptor antagonisten, iodophenpropit het beste onderscheid maakt tussen H3- en niet-H3 receptor bindingsplaatsen in de [^{3}H]-thioperamide verdringingsstudies.
Deze resultaten werden bevestigd in **hoofdstuk 5**, waarin de histamine H3 receptor, gebruikmakende van de H3 receptor antagonist [^{125}I]-iodophenpropit werd gekarakteriseerd in de hippocampus van de rat. Tot een concentratie van 1 μM, verdrong de H3 receptor agonist (R)-α-methylhistamine 30% van de totale binding van [^{125}I]-iodophenpropit, terwijl H3-antagonisten 50-60% van de gebonden [^{125}I]-iodophenpropit verdrongen. Deze gegevens leveren opnieuw bewijs voor de binding van H3-receptor antagonisten aan een niet-H3 receptor bindingsplaats(en).
In een poging, voorafgaande aan de bovengenoemde studies, de histamine H3 receptor in humane hersenen van gezonde en schizophrenie patienten te karakteriseren (A. Alves-Rodrigues, ongepubliceerde data), was het mogelijk om de binding van 0.25 nM [^{125}I]-iodophenpropit te verdringen door H3 antagonisten zoals thioperamide, maar slechts een klein gedeelte van de binding kon verdrongen worden door de H3 receptor agonist (R)-α-methylhistamine (figuur 1).
Op basis van de gegevens van **hoofdstuk 4** en **5** is het aannemelijk dat een veel hogere dichtheid van cytochroom P450 isoenzymen ten opzichte van het aantal H3 receptoren in de humane hersenen de reden is voor de afwezigheid van verdringing van [^{125}I]-iodophenpropit door H3 receptor agonisten. De verdringingscurve van thioperamide verkregen in humaan weefsel, duidt op binding aan meer dan een bindingsplaats, zoals ook werd waargenomen voor [^{3}H]-thioperamide in de cerebrale cortex van de rat. Voorlopige studies lieten zien dat clozapine, een non-imidazol-verbinding, de enige H3 receptor antagonist is (van 8 verschillende verbindingen), die de [^{125}I]-iodophenpropit binding niet kon verdringen. Het gebruik van een aselectieve cytochroom P450 remmer zou de binding aan de niet-H3 receptor bindingsplaats in humane hersen membranen misschien kunnen verhinderen en het gebruik van

radioactief gelabelde H3 receptor antagonisten in bindingsstudies met humane hersenen mogelijk maken.



Figuur 1 Competitie door (R)-α-methylhistamine (open cirkels) en thioperamide (dichte cirkels) van de binding van 0.25 nM [^{125}I]-iodophenpropit in membranen van humane hersenen. Data van één represenatief experiment zijn weergegeven in triplo.

De histamine H3 receptor in de hippocampus.

De karakterisering van de histamine H3 receptor in de hippocampus van de rat in **hoofdstuk 5** toont de labelling van één enkele bindingsplaats door [^{125}I]-iodophenpropit. De binding van [^{125}I]-iodophenpropit aan hippocampus membranen werd verdrongen door histamine H3 receptor agonisten en antagonisten met affiniteiten, die vergelijkbaar waren met waarden gevonden voor de H3 receptor in de cortex van de rat. Zoals verwacht werd de [^{125}I]-iodophenpropit binding strereoselectief verdrongen door de (R) en (S) enantiomeren van de H3 receptor agonist α-methylhistamine. In **hoofdstuk 5** werd ook de functionaliteit van de H3 receptor in de hippocampus van de rat aangetoond; de electrisch gestimuleerde [^{3}H]-noradrenaline afgifte uit hippocampus slices kon effectief verlaagd worden door stimulatie van de H3 receptor. Er werden geen significante verschillen tussen de hippocampus en de cerebrale cortex gevonden voor de niveaus van maximale remming van electrisch gestimuleerde [^{3}H]-noradrenaline afgifte, alsmede tussen de S_2/S_1 ratio. Zowel de pD_2 waarden voor (R) and (S)-α-methylhistamine, als de pA_2 waarden voor thioperamide en burimamide waren vergelijkbaar in beide cerebrale regio's.

Na de karakterisering van de H3 receptor in de hippocampus van de rat werd in **hoofdstuk 6** gestart met studies naar de veranderingen in de dichtheid en functie van de histamine H3 receptor in de hippocampus van de rat bij veroudering. In twee jaar oude ratten werd een H3 receptor upregulatie van 95% waargenomen in de hippocampus, terwijl een afname van 35% van het aantal H3 receptoren in de cerebrale cortex werd waargenomen. In het striatum werden geen verschillen in H3 receptor dichtheid waargenomen.

De histamine niveaus in de hypothalamus vertoonden een toename van 23% en geen significante verschillen in de andere geteste hersengebieden. In functionele studies werd een lagere electrisch gestimuleerde afgifte van $[^3H]$-noradrenaline (S_1 en S_2) verkregen in de hippocampus. Zowel in de hippocampus als de cortex werd een significante afname van het maximale niveau van de H3 receptor gemedieerde remming van de $[^3H]$-noradrenaline release gevonden in de oude ratten. Bovendien werd in de hippocampus, een significante afname van de pD_2 waarde voor (R)-α-methylhistamine waargenomen. In oude ratten is de H3 receptor functionaliteit in de hippocampus dus duidelijk verminderd. Speculatief, kan met zeggen dat in de hippocampus een toename van het aantal receptoren, tot op zekere hoogte, een minder efficiente H3 receptor in oude ratten probeert te compenseren.

De in dit proefschrift beschreven studies hebben bijgedragen tot een beter begrip van de farmacologie en fysiologische veranderingen in expressie van de H3 receptor. De ontwikkeling van H3 receptor liganden, die niet binden aan de beschreven niet-H3 receptor bindingspaats(en) is van groot belang. Goede kandidaten voor dit soort liganden kunnen afgeleid worden van de verbindingen, zoals beschreven in hoofdstuk 2 en 3. Het bestaan van H3 receptor subtypen en liganden met verschillende affiniteiten voor eventuele centrale en perifere H3 receptoren, zoals de immepip analoga in hoofdstuk 3, zijn interessant in verband met eventuele therapeutische therapeutische toepassingen van H3 liganden. Design van H3 antagonisten die de bloed-hersen-barriere goed penetreren blijkt tot nu toe moeilijk. Dit komt waarschijnlijk door binding (en metabolisme ?) van de verbindingen aan cytochroom P450. Betere H3 receptor liganden, als radioactief gelabelde verbindingen voor bv. PET studies, zullen zeker bijdragen tot de opheldering van de veelbelovende rol van de histamine H3 receptor in hersenfuncties.

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...And using Violetta Parra's words,

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