UNIVERSITÀ DEGLI STUDI FIRENZE

DOTTORATO DI RICERCA IN FARMACOLOGIA, TOSSICOLOGIA E TRATTAMENTI INNOVATIVI

CICLO XXVI

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DIFFERENTIAL MODULATION OF THE BRAIN HISTAMINERGIC SYSTEM: EFFECTS ON COGNITION AND FEEDING BEHAVIOUR

Settore Scientifico Disciplinare BIO/14

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Anni 2011/2013

A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.
Marie Curie

Fermare la diffusione del sapere è uno strumento di controllo per il potere perchè conoscere è saper leggere, interpretare, verificare di persona e non fidarsi di quello che ti dicono. La conoscenza ti fa dubitare. Soprattutto del potere. Di ogni potere.
Dario Fo

RINGRAZIAMENTI

Per citare Jean de la Bruyère "*Non c'e nel mondo esagerazione più bella che la gratitudine*" ed io sono grato a tante persone che hanno contribuito, ciascuna a proprio modo, alla costruzione di questa conquista; purtroppo è difficile, in poche righe, ricordarle tutte quindi mi scuso in anticipo con chi ho dimenticato di nominare.

Per prima cosa vorrei ringraziare i miei tutori il Prof. Patrizio Blandina e la Prof.ssa Beatrice Passani che si sono dimostrati sempre attenti e disponibili. Li ringrazio per il modo in cui mi hanno accolto nel loro laboratorio, per avermi aperto le porte e avermi guidato attraverso il mondo della ricerca “istaminergica" che fino all'incontro con loro era per me un grande sconosciuto. Grazie per la vostra pazienza, soprattutto nei primi tempi, quando la mia conoscenza della lingua italiana era ancora limitata e comunicare era davvero molto difficile. Vi sono grato per gli insegnamenti, le conversazioni, l'amicizia, la fiducia in me e nel mio lavoro. Vorrei ringraziare la Prof.ssa Emanuela Masini (anche se mi ha soprannominato "frikketone" e "Gustavino"), la quale rappresenta per me un esempio di come si puo fare ricerca in modo serio ma anche divertirsi. Ringrazio anche le Professoresse Maria Grazia Giovannini e Anna Maria Pugliese per il loro incoraggiamento durante tutti questi anni.

Ringrazio il Dott. Leonardo Munari, a cui oltre che un collega considero un amico. Ha avuto un ruolo fondamentale nella mia decisione di trasferirmi in Italia e mi ha aiutato tanto non soltanto negli esperimenti, ma anche a capire come risolvere i molti problemi burocratici. Inoltre, posso dire senza ombra di dubbio che ci siamo divertiti nella notte fiorentina!

Vorrei anche ringraziare gli altri colleghi, Dott. Hayato Umehara, Lucas Canto de Souza e Roberta Fabbri. Un grazie particolare anche alle tesiste, o meglio "le mie ragazze": Alice Brogi, Viola Busoni, Giulia Langella, Silvia Bardazzi, Alessia Costa e Linda Pescatore per l'attenzione e la dedizione agli esperimenti e per la loro assistenza così importante nella realizzazione dei progetti sviluppati in questa tesi.

Ringrazio inoltre le Dott.sse Gabriella Stocca, Chiara Traini e Teresa Ed Dami per la nostra indubbia affinità, alla fine siamo profili vincenti, vero ☺? La mia gratitudine anche per le innumerevoli risate e i momenti belli che abbiamo

vissuto, sia dentro che fuori del CUBO. Un grazie particolare anche alla Dott.ssa Gilda Baccini (per me semplicemente "Gildi"): mia vicina, revisore grammaticale, compagna di avventure e di viaggi e, soprattutto, una vera amica.

Ringrazio anche il comitato di accoglienza "brasiliani a Firenze": Denilso, Leticia, Margarete, Eduardo, Kamila, Debora (o Nega, per gli intimi), Dudu Letti e la nostra mascotte Mahler, la cui amicizia e sostegno sono stati essenziali per superare le difficoltà iniziali e abituarsi al nuovo paese, i nuovi costumi e la nuova lingua.

Grazie alle persone speciali che ho incontrato nel corso degli anni e con cui mi sono divertito tanto: Michele Leccese, Michela Romano, Enrico Bianchini, Pietro Tacconi, Valentina Spinelli, Massimo Tognaccini, Eduardo Cabruja, Federico Maio. Un pensiero anche agli amici che, sebbene lontani, mi hanno incentivato e che, sono certo, saranno contenti di questo traguardo: Fernanda Ely, Fernanda Camozzato, Gisele Ribeiro, Mariana Lopes da Silveira, Simone Gnoatto, Leo Albuquerque, Raquel Bassani e Edimar.

Infine ringrazio la mia famiglia: i miei genitori, Rosalina e Luiz, all’inizio non erano troppo contenti dell'idea che io venissi a vivere cosi lontano, ma hanno comunque sempre creduto in me, mi hanno sostenuto incondizionatamente e mi hanno incoraggiato in tutte le mie decisioni. Molto di quello che sono oggi, lo devo all'esempio che ho ricevuto da voi. Ringrazio inoltre la mia sorella Raquel, mio cognato Gilmar e la mia nipotina Maria Antonia (che per ora mi conosce solo da fotografie). Grazie per il sostegno che mi avete sempre dato. Vi amo tutti!

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INTRODUCTION

In the beginning of the XX century, Sir Henry Dale and his colleagues at Wellcome Laboratories in London, while studying the effects of ergot extracts, identified histamine for the first time in nature. They then carried out a series of experiments to explore its biological actions and found that it had a stimulant effect on smooth muscle from the gut and respiratory tract, caused vasodepression, stimulated cardiac contractility and induced a shock-like syndrome when injected into animals. Almost simultaneously, the stimulation of acid secretion in the dog stomach caused by histamine was also recognized by Leon Popielski. In 1924, Sir Thomas Lewis described the classical “triple response” to histamine consisting of a red spot due to vasodilatation, a wheal as consequence of increased permeability and flare due to an axon reflex. (Parsons & Ganellin, 2006).

The evidence that histamine had an active role in allergy and anaphylaxis stimulated the search for compounds able to counteract its pathological effects. In 1936 Daniel Bovet and its colleagues working at the Institute Pasteur in Paris reported the first antihistamine compound that blocked the effects of histamine on guinea-pig ileum and protected the lethal effects of histamine-induced anaphylaxis (Parsons & Ganellin, 2006; Figueroa & Shankley, 2011). Some years later, after the discovery of propranolol, a β-blocker that revolutionized the treatment of hypertension, Sir James Black started to study gastric acid secretion. He postulated that histamine stimulates this process through a novel class of receptors, the histaminergic H2 receptors (H2Rs), and together with the chemists from SmithKline & French pharmaceutical company, modified the structure of histamine to create a series of analogues. The efforts of Dr. Black and his associates were rewarded in the 1970s when he and his team developed a clinically useful H2R antagonist cimetidine that became the world's first billion-dollar drug (Rubin, 2007).

The presence of histamine in the brain was first hypothesized by Kwiatkowski (1941) and White (1959), who demonstrated its formation and catabolism in the brain. In addition, the observation that antihistamines have a sedative effect suggested that histamine had relevant actions in the central nervous system (CNS). However, the failure to demonstrate histamine localization in the brain using *o*-phtalaldehyde fluorimetric assay in the 1960s greatly limited the understanding and acceptance of this neuronal system (Haas

et al., 2008). In the early 1980s two major discoveries convinced the scientific community of the existence of a central histaminergic system and contributed to the understanding of its many functions: Jean-Charles Schwartz with his group in Paris proved with pharmacological experiments the existence of a new histaminergic receptor (the H3 autoreceptor) in neurons (Arrang *et al.*, 1983). In the next year Pertti Pannula and Takehiko Watanabe independently developed antibodies to identify histaminergic neurons and demonstrated their localization in tuberomamillary nucleus (TMN) of the posterior hypothalamus with projections to almost all parts of the brain (Panula *et al.*, 1984; Watanabe *et al.*, 1984). A plethora of neuropharmacological and behavioral studies ensued and it soon became clear that brain histamine affects a variety of homeostatic regulatory function such as circadian rhythms, neuroendocrine secretion, food and drinking intake, but also more complex brain functions such as arousal, emotionality and cognition (Munari & Passani, 2011). The central histaminergic system quickly became an attractive target for the development of new centrally active drugs. Indeed a series of clinical studies evaluating the efficacy of histaminergic drugs for the treatment of narcolepsy, obesity and cognitive impairments associated neurodegenerative diseases are under way (Passani & Blandina, 2011).

The Central Histaminergic System

Anatomy and Morphology

Histaminergic neurons have been found in a variety of vertebrate brains: fish, turtle, frogs, snake, bird, rodents, primates, etc. In all of these species, including humans, the histamine-containing cell bodies are localized solely in the tuberomamillary nucleus (TMN) of the posterior hypothalamus (Haas & Panula, 2003). From the TMN, histaminergic fibers project widely to almost all the central nervous system through ascending and descending efferent and afferent pathways, this way nearly all CNS areas contain some histaminergic fibers. The density of innervation is heterogeneous and follows closely the tissue concentrations of histamine and its biosynthetic enzyme found throughout the brain (Hough *et al.*, 1984). The highest densities are found in several hypothalamic nuclei, the medial septum, the nucleus of the diagonal band and ventral tegmental area. Substantial innervation is found also in the hippocampus and thalamus. Moderate densities are found in cerebral cortex, amygdala,

striatum and substantia nigra. Most areas of the brainstem, as well as retina, cerebellum and spinal cord contain only a small number of fibers (Inagaki *et al.*, 1988b; Panula *et al.*, 1990). TMN histaminergic cells also receive innervation from several brain regions, including the infralimbic prefrontal cortex, the septum/diagonal band complex, the preoptic area and the hypothalamus (Leurs *et al.*, 2012).

The human histaminergic system is quite extensive with around 64000 neurons within and around the TMN (Panula *et al.*, 1990). In the rat are found nearly 4500 histaminergic neurons subdivided in 5 clusters (E1-E5) (Figure 1). The subdivisions are bridged by scattered neurons in keeping with the concept of one continuous cell group that becomes dispersed during development (Watanabe *et al.*, 1984). Tracing studies have, so far, revealed a low level of topographic organization (Inagaki *et al.*, 1988a). The TMN in the mouse is less compact and contains fewer and smaller neurons (nearly 3000) than in the rat (Parmentier *et al.*, 2002). The morphological characteristics of histaminergic perikarya are similar through the species: bipolar or multipolar cells with large somata with a well-developed Golgi apparatus and many mitochondria (Haas *et al.*, 2008). The TMN is the only source of neuronal histamine in the adult vertebrate brain, but a number of other substances and enzymes are also expressed within TMN neurons, including: glutamate decarboxylase, GABA, GABA-transaminase, adenosine deaminase and monoamino oxidase-B, as well as neuropeptides such as methenkephalin, galanin and thyrotropin-releasing hormone (Haas *et al.*, 2008; Schwelberger *et al.*, 2013).

Synthesis and Metabolism

Although histamine is present in plasma, it does not penetrate the blood-brain barrier, and thus histamine concentrations in the brain must be maintained by synthesis. Histamine is formed in one step decarboxylation of the amino acid L-histidine, a process catalyzed by the enzyme histidine decarboxylase (HDC E.C. 4.1.1.22) which is a pyridoxal 5'-phosphate-dependent enzyme highly conserved thought the animal kingdom (Schwelberger *et al.*, 2013). The rate of histamine synthesis is determined by the bioavailability of the precursor L- histidine, which is transported into the brain by a saturable and energy-dependent mechanism (Leurs *et al.*, 2012). Newly synthesized neuronal histamine is carried into

neuronal vesicles by exchange of two protons through the vesicular monoamine transporter VMAT2, consequently the presence of HDC in neuronal cells is associated with the presence of VMAT2 (Weihe & Eiden, 2000).

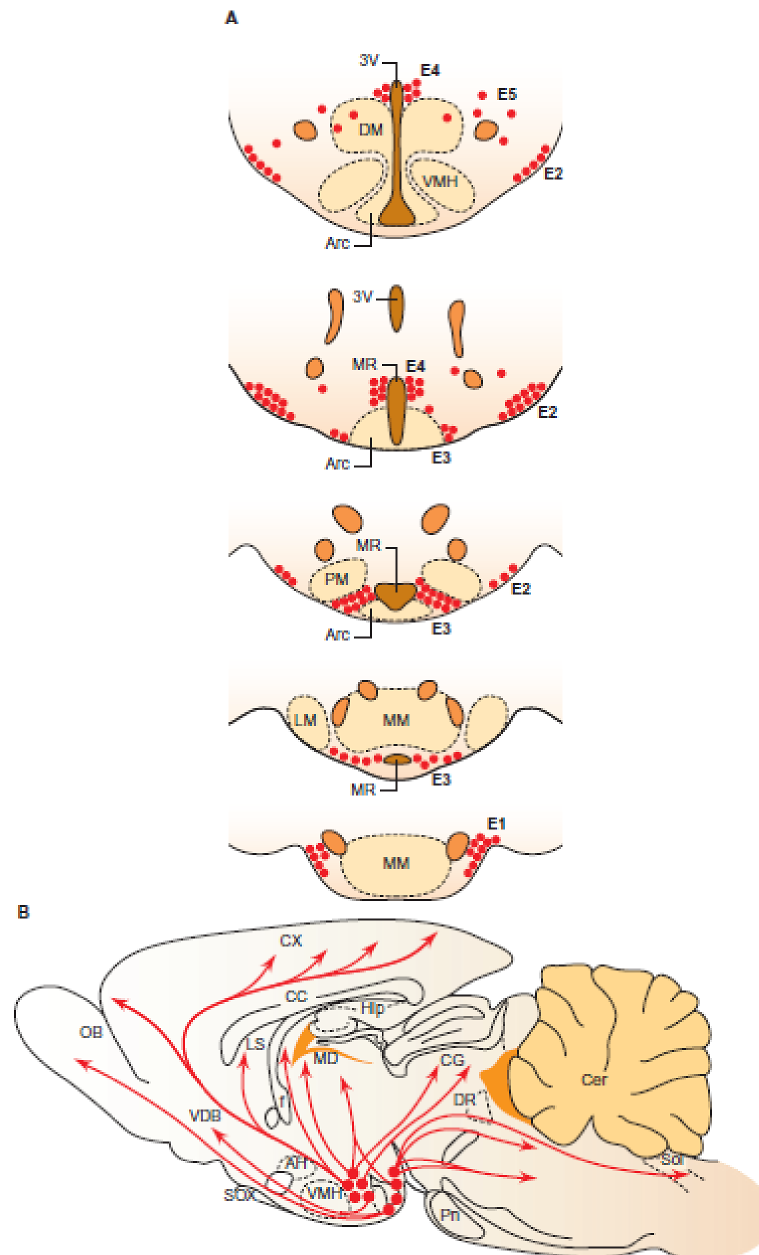

Figure 1. The histaminergic system of the rat brain. (A) Orizontal sections through the posterior hypothalamus showing the location of histaminergic neurons. Arc, arcuate nucleus; DM, dorsomedial nucleus; LM, lateral mamillary nucleus; MM, medial mamillary nucleus; MR, mamillary recess; PM, premamillary nucleus; 3V, third ventricle; VMH, ventromedial hypothalamic nucleus. (B) A sagittal view illustrating the major ascending and descending fiber projections. AH, anterior hypothalamus; CC, corpus callosum; Cer, cerebellum; CG, central gray; CX, cerebral cortex; DR, dorsal raphe; f, fornix; Hip, hippocampus; LS, lateral septum; MD, mediodorsal thalamus; OB, olfactory bulb; Pn, pontine nuclei; Sol, nucleus of solitary tract; SOX, supraoptic decussation; VDB, vertical limb of the diagonal band; VMH, ventromedial hypothalamic nucleus. (From Leurs *et al.*, 2012)

Both *in vitro* and *in vivo* studies showed that depolarization of nerve terminals activates the exocytotic release of histamine by a voltage- and calcium-dependent mechanisms (Kukko-Lukjanov & Panula, 2003). Unlike terminals from other amine transmitters, so far there are no evidences of a high-affinity uptake system for histamine on histaminergic nerve terminals (Schwelberger *et al.*, 2013).

In mammals there are two alternative routes for inactivation of histamine: methylation of the imidazole ring at the tele position by histamine N-methyltransferase (HNMT) and oxidative deamination of the primary amino group by diamine oxidase (DAO) (Schwelberger *et al.*, 2013). In the vertebrate brain, histamine metabolism occurs in the extracellular space, predominantly by methylation using the methyl donor S-adenosyl-L-methyionine, generating tele-methylhistamine, which undergoes oxidative deamination through a monoamine oxidase (MAO-B) to tele-methyl-imidazoleacetic acid (Haas & Panula, 2003; Leurs *et al.*, 2012). Both reactions convert histamine into products that are virtually inactive at histamine receptor.

Histaminergic Receptors

Once released, histamine activates both presynaptic and postsynaptic receptors. So far four receptor subtypes were identified, namely: H1, H2, H3 and H4. They are all linked to G-proteins and all of them have been found inside and outside the central nervous system. The histaminergic receptors differ in their signal transduction pathways, distribution, functionality and pharmacological properties of their agonists and antagonists.

Histaminergic H1 Receptors (H1R): The human H1R is a 487-amino acid peptide weighting 56 kDa (Haas *et al.*, 2008), its activation leads to stimulation of a pertussis toxin-insentive G protein, $G\alpha_{q/11}$, that stimulates phospholipase C (PLC) with the subsequent generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). These two mediators are known to elevate intracellular Ca^{2+} concentrations and activate phosphokinase C (PKC), respectively. Activated PKC facilitates capacitive Ca^{2+} entry through voltage-dependent calcium channels (VDCC). H1R also can activate phospholipase A_2 (PLA_2) with the subsequent release of arachidonic acid and its metabolites trough pertussis

toxin-sensitive G_i/G_o (Leurs *et al.*, 1995). Other effector pathways of the H1R include production nitric oxide (NO) and cGMP through $[Ca^{2+}]$i-dependent NO synthases, and NO-dependent guanylate cyclases, respectively. Importantly, H1R activates also AMP-kinase thus positively modulating the cAMP synthesis (Haas *et al.*, 2008).

H1R are found throughout the whole body and nervous system with considerable variation among species. Particularly high densities of the H1R are found in brain regions concerned with neuroendocrine, behavioral, and control of the nutritional state, like the hypothalamus, aminergic and cholinergic brainsteam nuclei, thalamus, and cortex. In the human brain highest concentrations were found in cerebral cortex and in infralimbic structures (Martinez-Mir *et al.*, 1990; Haas *et al.*, 2008). Using functional imaging techniques it was found that H1R binding was lower in Alzheimer's disease, schizophrenia (Iwabuchi *et al.*, 2005) and depression (Kano *et al.*, 2004) patients as compared with age-matched healthy controls.

Genetic disruption of the gene encoding for the H1R in mice, results in impairments on episodic, procedural, spatial and working memories (Dere *et al.*, 2008; Zlomuzica *et al.*, 2009); reduced novelty-induced arousal (Zlomuzica *et al.*, 2008); shorter latency for initiating and prolonged durations of non-rapid eye movement (NREM) sleep episodes and decreased number of transitions between NREM sleep and wakefulness (Huang *et al.*, 2006), age-dependent susceptibility to status epilepticus (Kukko-Lukjanov *et al.*, 2012) and altered response to feeding-related peptides such as Orexin-A, Neuropeptide Y and Leptin (Masaki *et al.*, 2001b; Ishizuka *et al.*, 2006). Classic antihistamines acting at H1R have well-known sedative properties. Many other drugs like antidepressants and antipsychotics bind to H1R that supposedly cause side effects such as increased body weight (Haas *et al.*, 2008).

Histaminergic H2 receptors (H2R): The H2R is a 40 kDa, 359 amino acid peptide derived from an intronless gene. This receptor show only 40% of homology with the H1R (Leurs *et al.*, 1995) but exhibit strong sequence homology (83-95%) among species: guinea pig, mouse, rat, and dog (Traiffort *et al.*, 1995; Haas *et al.*, 2008). H2R is coupled to $G\alpha_s$ proteins. Activation of this receptor stimulates adenylyl cyclase and increase intracellular cAMP, which

activates protein kinase A (PKA) and the transcription factor CREB, key regulators of neuronal physiology and plasticity (Haas *et al.*, 2008; Passani & Blandina, 2011). Additional transduction mechanisms including activation of PLC, increased intracellular Ca^{2+}, inhibition of PLA_2 and release of arachidonic acid, which likely account for the opposing physiological responses elicited by H1R and H2R in many tissues (Haas *et al.*, 2008; Leurs *et al.*, 2012).

Both neuronal and non-neuronal cells of the brain express H2Rs. Distribution of these receptors in the rodent brain is widespread indicating that the H2R mediates a large number of postsynaptic actions of histamine. High density of H2R is found in the basal ganglia, amygdala, hippocampus and cortex (Traiffort *et al.*, 1995; Vizuete *et al.*, 1997). Immunohistochemistry studies showed colocalizations of H1R and H2R in some brain areas, indicating synergistic interactions between these receptors subtypes (Garbarg & Schwartz, 1988).

Mice deficient in H2R function exhibit selective cognitive deficits with impairment in hippocampal LTP (Dai *et al.*, 2007); enhanced response to the antinociceptive effect induced by morphine and orexin A (Mobarakeh *et al.*, 2005; Mobarakeh *et al.*, 2006) abnormal morphological features on the gastric mucosa (Kobayashi *et al.*, 2000); increased ghrelin expression and gastric secretion accompanied with increased food intake and body-weight gain (Arakawa *et al.*, 2007). Cimetidine and ranitidine are H2R antagonists widely prescribed for the therapy of gastric disorders (Yu et al. 2011). Tricyclic antidepressants also have H2R antagonistic properties (Traiffort *et al.*, 1991). Some reports suggest the efficacy of famotidine, another H2R antagonist, in schizophrenia patients (Rosse *et al.*, 1996; Meskanen *et al.*, 2013).

Histaminergic H3 Receptor (H3R): Discovered in 1983 by Jean Charles Schwartz and his group the histamine H3R is a 70 kDa 445 amino acid peptide. Although the genes encoding the H1 and H2 receptors have been known since 1991, it was just on 1999 that the third histaminergic receptor was cloned (Lovenberg *et al.*, 1999). Analysis of the H3R protein sequence showed low homology with the H1 or H2 receptors: only 21 and 22%, respectively (Leurs *et al.*, 2012). The H3R gene from several species contains at least three introns, consequently as result of alternative splicing, various isoforms have been

identified in humans, rat, guinea pig and mouse (Hancock *et al.*, 2003). Currently, at least 20 different human H3R mRNAs have been identified, but it is not known whether all these potential isoforms are actually expressed in the body. Differences in ligand-binding characteristic and signaling properties have been described, but the importance of these signaling variances for the histamine-mediated actions are not know (Bakker, 2004).

A pertussis toxin-sensitive inhibition of cAMP accumulation and subsequent reduction of PKA activity in response to H3R agonists has been observed in a variety of transfected cells, indicating that the H3R negatively regulates adenylate cyclase activity via $G\alpha_{i/o}$ proteins (Bongers *et al.*, 2007a). The H3R mediated regulation of HDC activity is also controlled via de cAMP-adenylate cyclase-PKA pathway (Gomez-Ramirez *et al.*, 2002). In transfected cells, H3R-mediated activation of $G\alpha_{i/o}$ proteins has also been reported to: modulate PLA_2 activity and consequent arachidonic acid release (Morisset *et al.*, 2000), modulate the Na^+/H^+ exchanger (Silver *et al.*, 2001) and inhibit Ca^{2+} influx (Silver *et al.*, 2002; Bongers *et al.*, 2007a). The H3R has also been reported to activate the mitogen activated protein kinase (MAPK) pathway in both transfected cells and rat brain (Drutel *et al.*, 2001; Giovannini *et al.*, 2003). Moreover, H3R stimulation induces phosphorylation of the AKT/GSK-3β axis in transfected cells, primary rat cortical neurons and rat striatal slices (Bongers *et al.*, 2007b; Mariottini *et al.*, 2009) (Figure 2). On histaminergic neurons, H3R modulates histamine synthesis and release whereas on non-histamine neurons the H3R regulates release of other neurotransmitters including ACh, dopamine, GABA, serotonin and peptides (Haas *et al.*, 2008).

The CNS contains the great majority of H3R, although they can also be found in the periphery, such as heart, lung, and intestine (Hancock *et al.*, 2003). In rodents, H3R expression is observed in cerebral cortex, hippocampus, amygdala, nucleus accumbens, globus pallidus, striatum, and hypothalamus (Leurs *et al.*, 2005). In humans H3R expression is prominent in basal ganglia, globus pallidus, hippocampus and cortex (Martinez-Mir *et al.*, 1990). Loss of H3R function in knockout mice is associated with increased histaminergic transmission and reduction of slow wave sleep, reduced locomotion, hyperphagia, late-onset obesity, increased insulin and leptin levels, and an increased severity of neuroinflammatory diseases (Toyota *et al.*, 2002; Tokita *et*

al., 2006; Teuscher *et al.*, 2007; Gondard *et al.*, 2013).

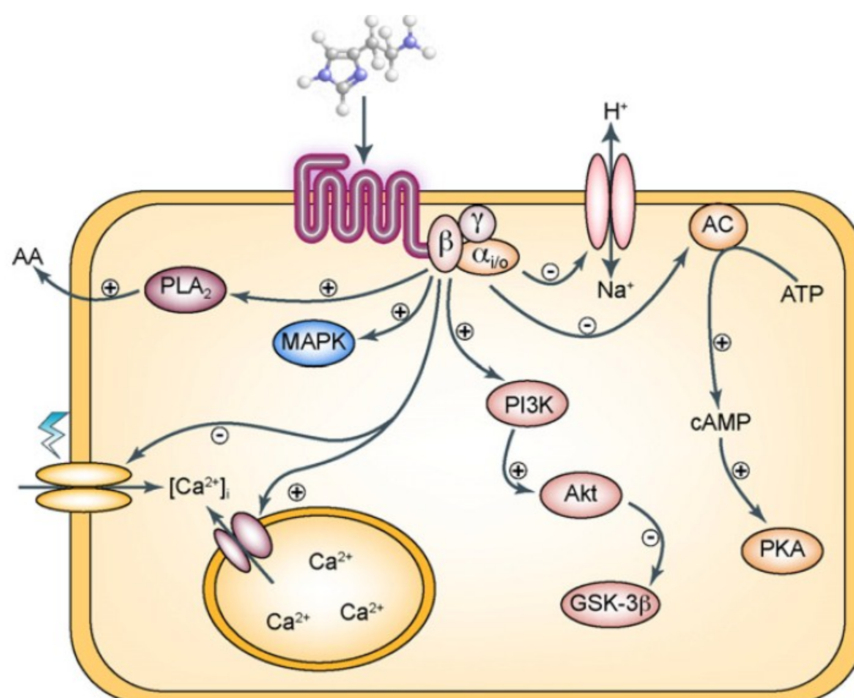

Figure 2: A schematic representation of the H3R-mediated signal transduction. The H3R has been shown to modulate several signal transduction pathways including the inhibition adenylyl cyclase (AC), mitogen-activated protein kinase (MAPK), activation of phospholipase A_2 (PLA_2), intracellular calcium mobilization, activation of the Akt/GSK-3β axis and inhibition of the Na^+/H^+ exchanger (From Bongers *et al.*, 2007a).

An important and very interesting feature of the H3R, from the pharmacological point of view, is its high degree of constitutive or spontaneous activity (Morisset *et al.*, 2000). As a G-protein-coupled receptor, the H3R is an allosteric protein that can adopt various conformations in equilibrium and the spontaneously active state leads to constitutive activity. It has been suggested that the potency of various H3R antagonists might depend on their intrinsic activity as inverse agonists. This discovery is important for drug development because the ability to compete with constitutively active H3R states might have important therapeutic implications. For instance, if pathological conditions change the balance between the various H3R conformations, therapeutically effective compounds could be selected depending on their intrinsic activity (Morisset *et al.*, 2000; Passani & Blandina, 2011). Currently, several H3R antagonist/inverse agonists are in phase II and phase III clinical trials for

potential treatment of narcolepsy, cognitive impairments associated with Alzheimer's disease and Parkinson's disease, schizophrenia and attention deficit hyperactivity disorder and obesity (Passani & Blandina, 2011; Schwartz, 2011b; Tiligada *et al.*, 2011).

Histaminergic H4 Receptors (H4R): The H4 receptor is the newest member in the histaminergic receptor family, showing 37% sequence homology with the H3R and around 19% with the H1 and H2 receptors. This receptor is a 390 amino acid protein and, as the previous, is a G-protein-coupled receptor (Leurs *et al.*, 2009). Following the identification of the human H4R, the cDNA sequence of mouse, rat, guinea pig, pig, dog and monkey H4Rs have been reported and functionally expressed (Liu *et al.*, 2001; Oda *et al.*, 2002; Oda *et al.*, 2005). The monkey H4R shows a high sequence homology (92%) with the human orthologue (Oda *et al.*, 2005). Less percentage of homology, from 67% to 72%, was found between human H4R and the other species described (Liu *et al.*, 2001; Oda *et al.*, 2002; Oda *et al.*, 2005; Jiang *et al.*, 2008; Leurs *et al.*, 2009).

The H4Rs are coupled to pertussis toxin-sensitive $G\alpha_{i/o}$ proteins, thereby inhibiting forskolin-induced cAMP and eventually the modulation of transcription of genes regulated by cAMP-responsive elements in cell lines recombinantly expressing the H4R (Oda *et al.*, 2000; Zhu *et al.*, 2001). In transfected HEK-293 cells, activation of H4R stimulates [^{35}S]GTPγS binding and MAPK phosphorylation via a pertussis toxin-sensitive pathway (Morse *et al.*, 2001). Activation of endogenously expressed H4R in eosinophils and mast cells leads to pertussis toxin-sensitive calcium mobilization (Buckland *et al.*, 2003), via the activation of PLC (Hofstra *et al.*, 2003). Initially the H4R mRNA was found in eosinophils, T-lymphocytes, neutrophils, mast cells, bone marrow, spleen, heart, lung and kidney. More recently, it was demonstrated the presence of H4R in the mammalian CNS. Transcripts of H4R were found in several regions of human CNS, including spinal cord, hippocampous, cortex, thalamus and amygdala (Leurs *et al.*, 2009). Using a combination of immunohistochemical and eletrophysiological techniques (Connelly *et al.*, 2009) demonstrated that H4R are functionally expressed in distinct deep laminae in the human cortex, and mouse thalamus, hippocampus and cortex.

The high density of H4R in haematopoietic lineage suggests a role in

inflammation and allergy (Tiligada *et al.*, 2011), but H4R activity has also been linked with rheumatoid arthritis (Ikawa *et al.*, 2005), colon cancer (Cianchi *et al.*, 2005) and breast cancer (Medina *et al.*, 2011) and recently also with neuroinflammation (Passani & Ballerini, 2012). Therefore, the H4R has potential as a new drug target in a number of therapeutic areas.

Brain functions regulated by the central histaminergic system

Pharmacological studies in intact and histamine-deficient animals as well as humans link brain histamine with homeostatic brain functions such as behavioural state, biological rhythms, body weight, energy metabolism, thermoregulation, fluid balance, stress and reproduction. The central histaminergic system is also involved in the control of high brain functions as aversion, pleasure and reward, memory and cognition (Haas *et al.*, 2008). In the following sessions some of the brain functions modulated by the histaminergic action are reviewed. The specific role of the histaminergic system in learning and memory as well as energy metabolism and food control will be further discussed in Chapters II and III, respectively.

Sleep and Wakefulness

In the mammalian brain, a small number of cell groups in the brainstem, hypothalamus, and basal forebrain are crucial for the regulation of sleep and wakefulness. Among these cell groups, the histaminergic system is one of the most important neuronal systems for maintaining arousal (Luo & Leung, 2011). Several experimental observations support the hypothesis that the histaminergic system constitutes a major wake-promoting system. Direct electrophysiological recordings from cats showed that the activity of histaminergic neurons is high during waking and low or absent during sleep (Lin *et al.*, 1988), and their firing rate changes with the behavioral state (Weiler *et al.*, 1998). Experiments in rats showed that histamine release in the central nervous system is positively correlated with wakefulness and the histamine released is four times higher during wake episodes than during sleep episodes (Liu *et al.*, 2011; Zant *et al.*, 2012). The importance of histaminergic neurons in maintaining the awake state when challenged by environmental demands was demonstrated in mice lacking

HDC. These animals show normal 24 hours sleep and wake amounts under undisturbed conditions but a striking inability to stay awake in novel environments, along with slowing of electroencephalogram (EEG) activity, wake fragmentation, and increased REM sleep (Parmentier *et al.*, 2002; Anaclet *et al.*, 2009).

Wakefulness is a quite complex process maintained by the interactions among, or coordinated actions of the ascending arousal system (AAS), a wake-promoting system containing histaminergic, cholinergic, serotonergic, adrenergic, and orexinergic neurons. Each cell group contributes in a unique way to the onset or maintenance of wakefulness (reviewed in Passani *et al.*, 2007). Histamine, acting via H1 and H2 receptors, increases the responsiveness of lateral geniculate neurons to excite inputs and promotes tonic firing of thalamocortical neurons (McCormick & Williamson, 1991). Activation of H1Rs also elicits excitation of cholinergic neurons of the basal forebrain and pedunculopontine nucleus, noradrenergic neurons of the locus coeruleus, and serotonergic neurons of the dorsal raphe. In contrast, activation of H3R inhibits wakefulness, by reducing TMN activity and release histamine, acetylcholine and monoamines (Esbenshade *et al.*, 2008; Benarroch, 2010a; Zant *et al.*, 2012).

Among the interactions between histamine and the other neurotransmitters that form the AAS, undoubtedly, histaminergic and orexinergic systems are the better studied. A direct synaptic connectivity has been reported between orexin nerve terminals and histaminergic cells in the TMN that express a high density of orexin-2-receptors (Peyron *et al.*, 1998). A recent study found that prior treatment with alpha-fluoromethylhistidine (αFMH), a histamine synthesis inhibitor, decreased by 48.8% the amount of wakefulness caused by orexin-B. This observation supports the hypothesis that the waking effect of orexin-B strongly depends on the activation of histaminergic neurotransmission (Yasuko *et al.*, 2010).

Insomnia is one of the most common disturbances of the sleep-wake cycle, whereas other pathologies such as hypersomnia and narcolepsy are less common. Narcolepsy, a sleep disorder characterized by excessive daytime sleepiness, cataplexy and narcoleptic episodes; lifestyle (e.g. nocturnal jobs), and identified mental and neurological disorders, such as Alzheimer’s disease, depression and Parkinson’s disease, are associated with hyper somnolence.

Insomnia is usually treated with benzodiazepines, whereas narcolepsy and sleepiness are currently treated mainly with wakefulness-promoting compounds, like modafinil, and amphetamines that modulate the dopaminergic system (Korotkova *et al.*, 2007). In this context, the H3R could represent a promising target to treat hypersomnia, because H3R antagonists show a remarkable ability to promote wakefulness in animals by increasing histamine release that in turn stimulates postsynaptic H1R, which are crucial for the regulation of the sleep/wake pattern (Lin *et al.*, 2011). Results from phase II clinical studies are becoming available and confirm the validity of H3R antagonists to counteract somnolence and vigilance deficiency of diverse pathological origins. Pitolisant was the first H3R antagonist/inverse agonist to be tested in clinical trials of sleep disorders. It significantly reduced both the number and duration of diurnal sleep episodes assessed with the Epworth Sleepiness Scale in a cohort of narcoleptic patients (Schwartz, 2011b). Other H3R antagonists such as JNJ-17216498, PF-03654746, GSK-189254 advanced to phase II clinical trials as wake-promoting agents in narcoleptic patients, but results have not yet been disclosed. All of these compounds show a better pharmacological profile than amphetamines, caffeine or modafinil, and are devoid of rebound effects and behavioural excitation (Passani & Blandina, 2011).

Hibernation

During hibernation, metabolic functions, movements and brain activity are reduced to a minimum for life maintenance. Although animals enter hibernation through sleep, hibernation itself is not considered to be a classical sleep state. Upon arousal from hibernation animals enter non-rapid eye movement sleep and display slow-wave activity, but they don't show sleep homeostasis (Heller & Ruby, 2004; Panula & Nuutinen, 2013). Sleep deprivation in rats is characterized by high histamine levels in the brain (Zant *et al.*, 2012). The same was observed in hibernating ground squirrels, as they showed high histamine and tele-methylhistamine brain levels as compared to those found in the brain of euthermic animals (Sallmen *et al.*, 1999). Also, increased H1R and H2R and decreased H3R densities were found during hibernation (Sallmen *et al.*, 2003a) and it was demonstrated that slow infusion of histamine into the dorsal hippocampus, markedly delays arousal from hibernation in these animals

(Sallmen *et al.*, 2003b). Although the importance of high histamine levels and turnover during hibernation is not fully understood, histamine protects rat hippocampal neurons from excitotoxicity (Kukko-Lukjanov *et al.*, 2006) and cultured hibernator neurons from cold-induced damage via agonistic action on H1R (Tamura *et al.*, 2006) which may contribute to explain why histamine levels are high during hibernation (Panula & Nuutinen, 2013).

Thermoregulation

Both core body temperature and brain histaminergic activity exhibit circadian rhythmicity (Mochizuki *et al.*, 1992). The preoptic area (PO) of the anterior hypothalamus a region that contains temperature sensitive neurons appears to be the main locus in which histamine affects body temperature. When injected in this region, histamine or SKF91488, a HNMT inhibitor that causes a local increase of histamine concentration, induced hyperthermia (Green *et al.*, 1976; Colboc *et al.*, 1982; Lundius *et al.*, 2010; Tabarean, 2013). Hyperthermia, in turn, facilitates neuronal histamine release (Kanamaru *et al.*, 2001). Using single-cell RT/PCR it was demonstrated that H3Rs were present in GABAergic cells in the median portion of the preoptic area (MnPO) and their activation resulted in hyperthermia (Lundius *et al.*, 2010). The warm-sensitive preoptic GABAergic neurons that express H3Rs contribute to the inhibitory tone sent to thermoregulatory neurons present in the rostral raphe pallidus and dorsomedial hypothalamus. Thus, histamine, by activating H3Rs, causes a decrease in the frequency of the tonic firing of GABAergic cells resulting in diminished inhibition of downstream thermoregulatory neurons and increased thermogenesis. These findings are in agreement with the fact that transgenic mice lacking H3Rs display a lower body temperature (Takahashi *et al.*, 2002). Interestingly, H1Rs are expressed in glutamatergic neurons, but not in MnPO GABAergic cells (Lundius *et al.*, 2010; Tabarean, 2012) and their activation also induced hyperthermia of similar amplitude to that induced by histamine (Lundius *et al.*, 2010), however the mechanism of hyperthermia induced by H1Rs is still not completely understood.

Previous *in vivo* studies indicated a role for the H2R as well in regulating body temperature. These receptors are found in the caudal region of the MPO since bilateral microinjections of histamine into this nucleus induced

hyperthermia, whereas the H2R antagonist cimetidine antagonized the hyperthermia elicited by an intracerebroventricular administration of histamine in rats (Colboc *et al.*, 1982). Recently, (Tabarean *et al.*, 2012) observed that injections of H2R agonist dimaprit in the mouse MPO reduced body temperature. They also identified expression of the H2R in glutamatergic but not GABAergic neurons in this nucleus. Accordingly, the activation of this receptor did not affect the firing rate of identified GABAergic preoptic neurons but depolarized and increased the firing rate of a population of glutamatergic neurons giving further evidence for the role of a PO glutamatergic output in the control of thermoregulation.

Fluid Intake and Balance

The role of histamine in diuresis has been extensively studied and shows mechanisms independent of food intake regulation. Injection of histamine into the cerebral ventricles or into hypothalamus elicits drinking in rats (Leibowitz, 1973). Histamine induced depolarization of the vasopressin neurons in the supraotic nucleus (Smith & Armstrong, 1996) and evokes antidiuresis when administered within this nucleus (Bennett & Pert, 1974). Additionally, histamine and H1R or H2R agonists administered intracerebroventricularly stimulated vasopressin secretion, whereas infusion of H1R and H2R antagonists prevented the increase of vasopressin release induced by noradrenaline (Knigge *et al.*, 1999; Radacs *et al.*, 2006).

Prolonged (24 or 48 hours) dehydration increases synthesis and release of histamine and vasopressin in the hypothalamus (Kjaer *et al.*, 1994). Pharmacological blockade of histamine synthesis, H3R activation, or H1R and H2R antagonism strongly reduces dehydration-induced vasopressin release (Kjaer *et al.*, 1995). Indeed, acute functional blockade of central H3R is often demonstrated by blocking the dipsogenia response to selective H3R agonists (Fox *et al.*, 2005).

Reward and Addiction

The effect of brain histamine on reward is thought to be mainly inhibitory but it is still controversial. Initial studies demonstrated that histamine injected in the lateral ventricle or lateral hypothalamus suppressed brain self-stimulation

behaviour in rats and this effect was blocked by the H1R antagonist pyrilamine (Cohn *et al.*, 1973). Successive studies demonstrated that TMN lesions gradually increased electric hypothalamic self-stimulation (Wagner *et al.*, 1993), thus histamine was assumed to play a negative role in reward. Contradictory findings were soon described as αFMH infused into the TMN in order to deplete histamine unexpectedly decreased, rather than increased, self-stimulation behaviour (Zimmermann *et al.*, 1997) suggesting that amplification of reward induced by TMN lesions does not necessarily result from a depletion of histamine in the brain. Moreover, histamine injected into the caudal part of the Nucleus accumbens (NAcc) (Hasenohrl *et al.*, 2001) or in the medial septum (Zarrindast *et al.*, 2006) induces conditioned place preference (CPP) in rats. Pyrilamine and the dopaminergic D1 antagonist SCH 23390, but not ranitidine or the D2 antagonist sulpiride blocked the histamine-induced CPP (Zarrindast *et al.*, 2006).

Evidence strongly suggests that endogenous brain histamine inhibits the rewarding effects of morphine. Systemic injections of histidine attenuated the acquisition of morphine-induced CPP in mice (Suzuki *et al.*, 1995) and rats (Gong *et al.*, 2007a). Like histidine, carnosine (β-alanyl-L-histidine), a dipeptide that is transformed into histamine in the brain, inhibited morphine place preference in rats (Gong *et al.*, 2007b). Antagonism of the H3R with thioperamide also prevented morphine-induced CPP (Perez-Garcia *et al.*, 1999). In contrast, αFMH injections potentiated morphine-induced CPP, an effect that was antagonized by SCH23390 in mice (Suzuki *et al.*, 1995). Rats with bilateral TMN lesions also showed improved morphine-induced CPP. In these animals histamine content was decreased whereas dopamine turnover was increased in the ventrotegmental area (VTA) and the NAcc without changes on glutamate or GABA levels (Gong *et al.*, 2007a). In agreement with these studies, morphine-evoked CPP as well as morphine-induced increase in dopamine levels in the VTA and NAcc were higher in HDC-KO mice as compared to WT littermates (Gong *et al.*, 2010). Several H1R antagonists have been shown to potentiate CPP effects induced by opioids. Tripelennamine potentiates CPP for pentazocine and morphine (Suzuki *et al.*, 1991). Chlorpheneramine and ebastine (Suzuki *et al.*, 1990), but not cetirizine (Kamei *et al.*, 2003), a second generation H1R that poorly penetrates the blood-brain barrier, potentiated the

dihydrocodeine-induced CPP. The preference effects induced by clorpheneramine, ebastine and zolantidine in combination with opioids were blocked by SCH23390. Zolantidine, a H2R antagonist, also augmented the morphine-induced CPP (Suzuki *et al.*, 1995). Taken together these findings show that activation of the histaminergic system attenuates while inhibition potentiates opioid-induced place preference, moreover decreased endogenous histamine seems to enhance dopaminergic activity, which contributes to morphine rewarding effects (Brabant *et al.*, 2010).

To date, no study has convincingly demonstrated that the histaminergic system modulates cocaine reward. (Ito *et al.*, 1997a) found that an acute injection of cocaine increased histamine levels and HNMT activity in the striatum, NAcc, and amygdala, moreover, histidine treatment blocked wheel-running behaviour induced by cocaine in rats. Concurrent histamine exposure was effective in reducing cocaine consumption in animal models of high and low vulnerability to drug abuse (Holtz *et al.*, 2013). Conversely, a reduction of cocaine-induced hyperactivity was observed in HDC-KO as compared with wild type mice, however no differences in cocaine-induced CPP were found between genotypes (Brabant *et al.*, 2007). Thioperamide strongly potentiated cocaine-induced hyperlocomotion and CPP (Brabant *et al.*, 2005; Brabant *et al.*, 2006; Brabant *et al.*, 2009). The enhancement of cocaine-induced hyperactivity after thioperamide treatment was also observed in HDC-KO mice, indicating that increased release of histamine produced by blockade of H3 autoreceptors is not required for this effect. Importantly, plasma cocaine concentrations were higher in mice treated with thioperamide as compared with the animals that received cocaine alone (Brabant *et al.*, 2009). Thioperamide is an imidazole-based compound that strongly inhibits CYP-450 enzymes (Yang *et al.*, 2002), the primary enzymes involved in the cocaine metabolism (Poet *et al.*, 1996), thus a pharmacokinetic interaction between thioperamide and cocaine might explain, at least in part, the results described. In agreement with this hypothesis, clobenpropit, another imidazole-based H3R antagonist agonist that blocks CYP450 enzymes increased cocaine-induced hyperlocomotion in mice, whereas A-331440, a non-imidazole based compound, did not (Brabant *et al.*, 2009; Brabant *et al.*, 2010).

Evidence shows that experimental manipulations that decrease brain

histamine amplify stereotypies and hyperlocomotion induced by methamphetamine. Inversely, the behavioural effects induced by this drug were reduced when histaminergic transmission was increased. Systemic injections of histidine or metoprime, an HNMT inhibitor, or H3R antagonists (ciproxifan, ABT-239 and pitolisant) attenuated locomotor activating effects of methamphetamine (Itoh *et al.*, 1984; Morisset *et al.*, 2002; Fox *et al.*, 2005; Ligneau *et al.*, 2007a). In contrast αFMH potentiated methamphetamine hyperlocomotion (Itoh *et al.*, 1984). The same effect was observed in HDC-KO mice (Kubota *et al.*, 2002). In agreement with the locomotion experiments, histidine, the HNMT inhibitors metoprine and SKF91488 decreased, whereas αFMH potentiated stereotypic behaviour induced by methamphetamine (Joshi *et al.*, 1981; Ito *et al.*, 1997b). Histidine effects were blocked by pyrilamine and ketotifen (centrally acting H1R antagonist), but not by fexofenadine (peripherally acting H1R antagonist) or thioperamide (Ito *et al.*, 1997b; Kitanaka *et al.*, 2007; Kitanaka *et al.*, 2010). In line with these results, the H3R agonists (R)-α-methylhistamine, imetit and immepip increased methamphetamine-induced stereotypical behavior in mice (Kitanaka *et al.*, 2011). Results from self-administration and discriminative experiments go in the opposite direction since it was showed that thioperamide and clobenpropit increased methamphetamine self-administration and potentiated methamphetamine-induced increase in dopamine levels in the NAcc shell (Munzar *et al.*, 1998; Munzar *et al.*, 2004). Amphetamines are metabolized by liver P450 enzymes, hence these effects could be also related with a pharmacokinetic interaction, and thus further work is needed to clarify these observations.

Lintunen *et al.*, 2001 investigated the role of the histaminergic system in alcohol preferring rats, which were developed by selective outbreeding for differences in voluntary alcohol consumption. They found that alcohol-preferring rats display higher levels of brain histamine and tele-methylhistamine as compared with non-preferring rats. They also observed that thioperamide and clobenpropit decreased self-administration in alcohol-preferring rats, whereas (R)-α-methylhistamine and pyrilamine did not affect alcohol consumption. Recently, the same group showed that in HDC-KO mice the alcohol-evoked CPP was stronger than in wild type counterparts (Nuutinen *et al.*, 2010). The H3R antagonists JNJ-39220675 and JNJ-10181457 totally inhibited the CPP induced

by alcohol in wild type mice but not in HDC-KO mice, suggesting that endogenous histamine is required for the H3R-mediated inhibition of alcohol reward (Nuutinen *et al.*, 2011b; Vanhanen *et al.*, 2013). Interestingly, H3R-KO mice consumed significantly less alcohol compared to wild type mice and did not developed CPP induced by alcohol (Nuutinen *et al.*, 2011a).

Studies with alcohol and morphine suggest that targeting brain H3Rs could be a potential therapeutic tool for the treatment of drug dependence. However, considering that multiple-drug abuse is often observed in addicted individuals and the conflicting findings about the H3R antagonist effects in cocaine and methamphetamine addiction-related behaviours, more studies are needed to really show whether H3R antagonist would be beneficial in other types of drug addiction (Nuutinen *et al.*, 2012).

The role of the central histaminergic system in CNS diseases

Cognitive dysfunction and Alzheimer's disease (AD)

Initial studies demonstrated accumulation of neurofibrillary tangles, loss of large histaminergic neurons (Nakamura *et al.*, 1993), decreased metabolic activity (Salehi *et al.*, 1995) in the rostral TMN in AD patients. Diminished histamine levels in different brain areas including the hippocampus, hypothalamus frontal and temporal cortex (Panula *et al.*, 1998) was also observed in AD patients. Several other reports claimed, however, increase histamine levels in frontal, parietal, temporal cortex and occipital cortices, hypothalamus, hippocampus and other brain regions, but also in the serum and cerebrospinal fluid of AD patients (Cacabelos *et al.*, 1989; Cacabelos *et al.*, 1992; Fernandez-Novoa & Cacabelos, 2001). Recent studies confirmed that TMN neurons were significantly (57%) lost in AD, however, total HDC-mRNA expression levels was not significantly decreased (24%) in those patients (Shan *et al.*, 2012b; Shan *et al.*, 2012c). Accordingly it was recently found a slightly but not significantly lower level (22%) of tele-methylhistamine, the histamine metabolite that reflects histamine release, in the cerebrospinal fluid of AD patients (Motawaj *et al.*, 2010). This suggest that the significant loss of large TMN neurons in AD patients is largely compensated, but the mechanism underlying such functional compensation is not known and deserves further

studies (Shan *et al.*, 2013). It was also observed an increase in H3R- and HMT-mRNAs expression in the prefrontal cortex of AD patients (Shan *et al.*, 2012b; Shan *et al.*, 2013).

The potential therapeutic use of many H3R antagonists are being investigated in Phase I and Phase II clinical trials to determine their possible efficacy in improving cognitive deficits in Alzheimer's disease, attention-deficit hyperactivity disorder and schizophrenia (Kuhne *et al.*, 2011; Passani & Blandina, 2011). The H3R antagonist MK-3134 ameliorated scopolamine-induced decline in memory-related tasks in healthy volunteers and such effect was superior when the volunteers received MK-3134 in combination with donepezil, an acetylcholinesterase inhibitor (Cho *et al.*, 2011).

Schizophrenia

The first antipsychotics were developed as antihistaminic compounds. Owing to their sedative and modest therapeutic effects, they did not prove to be successful in the clinic. It is now known that the atypical antipsychotic drugs have an affinity for many different receptors, including those for serotonin, noradrenaline and histamine (Panula & Nuutinen, 2013). Indeed, clozapine is a potent partial inverse agonist of H1Rs, a weak and full inverse agonist of H2Rs and a moderate agonist of H3Rs and H4Rs, and the targeting of these receptors may contribute to the antipsychotic and sedative profile of the drug (Humbert-Claude *et al.*, 2012). H1Rs, H2Rs and H3Rs are abundantly expressed throughout the human cerebral cortex, in the hippocampus and in the dorsal thalamus, which innervates the cortex. These areas are all known to be involved in higher cognitive functions that are disturbed in schizophrenia.

In the brain of people with schizophrenia, various changes in the histaminergic system are found: the levels of tele-methylhistamine, are increased in the cerebrospinal fluid of individuals with schizophrenia (Prell *et al.*, 1995); H1R binding is reduced measured as positron tomography in the frontal and prefrontal cortex and in the cingulate gyrus, possibly as a consequence of the increased histamine levels and turnover (Iwabuchi *et al.*, 2005); H3R binding, as measured by receptor radioligand binding autoradiography, is increased in the dorsolateral prefrontal cortex but unchanged in the temporal cortex of patients with schizophrenia compared with those brain regions in healthy control subjects

(Jin *et al.*, 2009). These differences may reflect the structural abnormalities of the cortical network and changes in cellular composition that underlie the functional impairments in this disorder. Thus, both pharmacological and anatomical evidence supports the concept that histaminergic mechanisms are involved in cognition and its disturbances in this condition (Panula & Nuutinen, 2013).

On the basis of a published case report on the positive therapeutic effects of famotidine (an H2R antagonist) on the negative symptoms of schizophrenia (Kaminsky *et al.*, 1990), an open-label study of this drug was performed and the results indicated that patients treated with famotidine showed significant improvements regarding the negative symptoms (Rosse *et al.*, 1996). The results from the first double-blind, placebo-controlled, parallel group, randomized trial of famotidine for treatment-resistant schizophrenia, which involved 30 individuals with this condition, indicated that the drug improved patient outcomes on the positive and negative symptoms (Meskanen *et al.*, 2013). These results suggest that although the H2R has not been commonly seen as a promising drug target in schizophrenia, it may be a relevant target for antipsychotic medication. Indeed, recent reviews suggest that H3R antagonists could be helpful in combating the cognitive if not the psychotic symptoms associated with schizophrenia (Bonaventure *et al.*, 2007; Esbenshade *et al.*, 2008; Sander *et al.*, 2008; Passani & Blandina, 2011). The use of a drug with a combination of H3R- and H2R-binding properties might be a powerful approach in treatment-resistant schizophrenia (Panula & Nuutinen, 2013).

Parkinson's Disease (PD)

There are conflicting opinions about the neuronal histaminergic system alterations in Parkinson's disease. Increased density of histaminergic innervation with enlarged varicosities (Anichtchik *et al.*, 2000b) as well as augmented histamine levels (Rinne *et al.*, 2002) in the substantia nigra of PD patients were described. Higher histamine levels were also found in putamen and globus pallidus of those patients as compared with age-mated controls (Rinne *et al.*, 2002). An association of a functional polymorphism in the HNMT gene in large samples of PD patients is also reported (Agundez *et al.*, 2008; Palada *et al.*, 2012). This polymorphism in the exon 4 of the HNMT gene leads to change of

threonine to isoleucine at position 105 (Thr105Ile). Individuals heterozygous for Ile105 allele have 30% to 50% lower HNMT activity, while homozygous individuals have decreased enzyme activity of about 60% (Preuss *et al.*, 1998), supporting the increased histamine levels described. Moreover, individuals with PD often experience fragmented sleep and insomnia, these problems may be related to the abnormalities in the histaminergic system which is a key regulator of sleep-wake cycle (Panula & Nuutinen, 2013). On the other hand, (Shan *et al.*, 2012d) observed an abundant accumulation of Lewy bodies and Lewy neurites, two characteristic neuropathological lesions, in the tuberomamillary nucleus of PD patients. However, in contrast to the presumed degeneration of this nucleus due the presence of such lesions, no clear quantitative changes in the number of histaminergic cells (Nakamura *et al.*, 1996), as well as unchanged HDC activity and unaltered tele-methylhistidine levels in the cerebrospinal fluid of those patients were found (Shan *et al.*, 2012a; Shan *et al.*, 2013). These authors observed also an up-regulation of HNMT-mRNA in the substantia nigra and putamen from the PD patients.

The findings regarding the H3R expression in PD are also controversial: H3R-mRNA expression in the substantia nigra is reduced (Anichtchik *et al.*, 2001; Shan *et al.*, 2012a), while H3R radioligand binding in the striatum and substantia nigra is strongly increased (Anichtchik *et al.*, 2001). There are also reports of no significant differences in H3R binding between normal and PD patients in the same brain areas (Goodchild *et al.*, 1999).

Gilles de la Tourette syndrome (GTS)

This syndrome is characterized by the appearance, during childhood, of multiple involuntary movements and vocal tics. This syndrome shows also high comorbidity rates with other neuropsychiatric disorders such as obsessive compulsive disorder, attention deficit/hyperactivity disorder and autism (Robertson, 2008). Genetic evidence indicates that dysfunction of the histaminergic system may also have a role in GTS. (Ercan-Sencicek *et al.*, 2010) studying a family with a father and eight of his children affected with this syndrome found a rare coding mutation W317X in exon 9 of the HDC gene that was not found in any of the 3000 control individuals evaluated. The W317X mutation is predicted to result in a truncated protein, unfortunately the detailed

phenotype of the affected individuals was not reported. Recently (Karagiannidis *et al.*, 2013) studied variations across the HDC gene in a sample of 520 GTS nuclear families originating from Albania, Canada, Germany, Greece, Hungary, Italy, Poland and Spain and identified a strong over-transmission of two single-nucleotide polymorphisms (rs854150 and rs1894236) in intronic regions of HDC gene, providing further support for the role of the histaminergic hypothesis in GTS.

Based on these genetic findings that suggest diminished histaminergic neurotransmission (Hartmann *et al.*, 2012) reported a case of a young GTS male patient (17 years old) that experienced also narcolepsy characterized by excessive daytime sleepiness and dyssomnia. The patient received pitolisant at a starting dose of 5 mg/day that was gradually increased during the 9 months of treatment until the final dose of 40 mg/day. The daytime sleepiness decreased dramatically, whereas just a small reduction of tics scores was observed. The authors described these results as encouraging and point to the necessity of controlled clinical trials to confirm this observation.

Eating behaviour disorders

The association of neuronal histamine with food consumption became clear when it was observed that antidepressants and antipsychotics stimulate appetite and induce weight gain and that these drugs are potent H1R blockers (Russ & Ackerman, 1988; Deng *et al.*, 2010). In animals, tricyclic antidepressants which potently block H1R, such as doxepin (Orthen-Gambill, 1988) and amitriptyline (Ookuma *et al.*, 1990) increase food intake, whereas an injection of desipramine, which has a negligible H1R-blockade effect, does not affect food intake (Orthen-Gambill & Salomon, 1990). The weight gain liability of different second generation antipsychotics correlates well with their affinity for the H1R and estimated occupancy (reviewed in He *et al.*, 2013). (Kim *et al.*, 2007) reported that histamine significantly inhibited AMP-activated protein kinase (AMPK) activity in hypothalamic slices; in contrast, the H1R antagonist, triprolidine, stimulated AMPK activity. It is well known that hypothalamic AMPK links energy metabolic status and neurotransmitter/neuropeptides systems to regulate energy balance and body weight (reviewed in Lage *et al.*, 2008). Briefly, AMPK activation by phosphorylation directly inhibits acetyl-CoA

carboxylase activity, which is an important enzyme that converts acetyl-CoA to malonyl-CoA. This effect results in a decrease in malonyl-CoA, which is an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT1), and inhibits fatty acid synthesis. Decreased malonyl-CoA levels disinhibit CPT1 activity, which upregulates food intake partly by increasing fatty acid oxidation (Kola, 2008). Both *in vitro* and *in vivo* studies have found that clozapine, olanzapine and quetiapine, with high affinities for H1R and a high risk of weight gain induction, significantly increased hypothalamic AMPK phosphorylation (Kim *et al.*, 2007; Martins *et al.*, 2010; Sejima *et al.*, 2011). In contrast, antipsychotics with lower orexigenic effects as well as lower H1R affinitiy such as ziprasidone are less effective at increasing hypothalamic AMPK activity (Kim *et al.*, 2007). Importantly, the stimulatory effect of clozapine on hypothalamic AMPK was attenuated by histamine administration and was abolished in H1R-KO mice (Kim *et al.*, 2007).

Interesting in this regard is the result from a recent published double-blind placebo-controlled clinical trial in which it was observed that olanzapine-induced weight gain was significantly minor in patients treated with a combination of reboxetine + betahistine (a mixed H1R agonist and H3R antagonist) than in placebo-treated controls (Amrami-Weizman *et al.*, 2013). The betahistine + reboxetine weight-attenuating effect was higher than observed with reboxetine alone (Poyurovsky *et al.*, 2007).

The H3 receptor as an attractive drug target in the brain

The discovery of the H3R by Schwartz and his group in 1983 was greeted with considerable excitement as a very attractive CNS drug target and has generated intense research efforts in both academic and industrial laboratories to identify potent and selective H3R ligands. The H3R is localized primarily in the CNS with highest expression, in rodents, in the cerebral cortex, hippocampus, striatum and hypothalamus (Drutel *et al.*, 2001). Similarly, in humans H3R expression is prominent in basal ganglia, globus pallidus, hippocampus and cortex (Martinez-Mir *et al.*, 1990). Notably many of these brain regions are critical for cognition (cortex and hippocampus) and sleep and homeostatic regulation (hypothalamus) (Esbenshade *et al.*, 2006). The H3R exhibits several properties that contribute to its attractive profile as a drug target: H3R activation moderates

histamine synthesis and release from histaminergic neuron endings; H3R acts also as an heteroreceptor in non-histaminergic neurons and moderates the release of other neurotransmitters, including ACh, dopamine, GABA, serotonin and peptides (reviewed in Passani *et al.*, 2004). From a therapeutic point of view, agents with multiple and complementary modes of action are more likely to show broad-based efficacy against core and comorbid symptoms (Passani & Blandina, 2011).

At the beginning of the 80s a collaboration between the groups of Jeanne-Marie Lecomte at Bioproject, Jean-Charles Schwartz at Institut National de la Santé et de la Recerche Médicale in Paris and medicinal chemistry groups of Max Robba, at the University of Caen and Walter Schunack at the University of Berlin was established to develop and characterize H3R ligands. The starting point was the structure of histamine itself, because is the natural agonist of this receptor. In few years, two main tools were derived from this program: the first, (R)-α-methylhistamine, a potent H3R agonist. This compound was used in many pharmacological studies, but its use *in vivo* is limited by its high basicity and hydrophilicity, extensive metabolism and low bioavailability (Schwartz, 2011b). Also selectivity issues hampered the use of (R)-α-methylhistamine, because it shows cardiovascular effects mediated by α2R and bronchoconstriction thought H1R activation (Leurs *et al.*, 2005).

The second, thioperamide, had been used as a reference H3R antagonist for almost two decades and many preclinical studies were carried out with this compound because it showed a high affinity for the rat and the human H3R (Schwartz, 2011b). Thioperamide shows high affinity also for the H4R and α2R (Esbenshade *et al.*, 2003). Sequentially more selective compounds (proxifan, ciproxifan, clobenpropit, etc) were synthetized, but nowadays these compounds are mainly used as reference structures because their imidazole basic ring holds diverse potential problematic issues: imidazole compounds potently bind at the heme iron of cytochrome P450 enzymes (CYP450) and when co-administered with other drugs, this may lead to drug–drug interactions and therefore, undesired side effects. Another point of concern is that imidazole is a strong hydrogen bond acceptor and donor, which reduces oral bioavailability and hampers brain penetration (Leurs *et al.*, 2005). The next step on the H3R antagonists development was the synthesis of new compounds changing the

imidazole ring for a variety of secondary and tertiary amines, partly in form of aliphatic heterocycles. This approach resulted in more potent and selective compounds with reduced pharmacokinetic interactions (Sander *et al.*, 2008).

These new H3R ligands were then extensively tested in several animal models and there is a growing evidence of its efficacy in increasing vigilance, improve memory and cognition and reduce food intake. Currently, several H3R antagonists/inverse agonists are under Phase II and III clinical trials for the potential treatment of narcolepsy, cognitive impairments associated with Alzheimer's and Parkinson's diseases, schizophrenia and attention deficit/hyperactivity disorder, as well as anti-obesity drugs (Passani *et al.*, 2011; Tiligada *et al.*, 2011)

GENERAL AIMS

The somata of histamine-containing neurons are localized in a discrete brain region in the posterior hypothalamus, the tuberomamillary nuclei (TMN), but in opposition to other aminergic systems, retrograde tracers injected into different CNS regions labelled histaminergic cell bodies scattered through the TMN without a strict topographical pattern (Kohler *et al.*, 1985). This observation led to consider that histaminergic neurons constitute a homogeneous cell group acting as a regulatory network for whole brain activity through diffuse projections rather than processing specific functions (Wada *et al.*, 1991). However, in the last decade our group and others demonstrated that the histaminergic system is organized into distinct functional circuits and evidence has accumulated showing the functional heterogeneity of histaminergic neurons (Blandina *et al.*, 2012). Our hypothesis is that this heterogeneity contributes to the diverse roles played by brain histamine in feeding homeostasis, arousal and cognition. To test our hypothesis we used a combination of *in-vivo* microdialysis, quantitative immunological techniques and behavioural tests. Our general aims were: (i) to investigate if and how an H3R antagonist modulate the activity of selective populations of histaminergic neurons; (ii) to investigate the role of the central histaminergic function in memory and feeding behavior.

In the first chapter we describe the results of experiments aimed at studying the functional heterogeneity of the histaminergic system by monitoring the effects of the H3R antagonist/inverse agonist ABT-239 on histamine release and neuronal activation in selected histaminergic projections areas in the rat brain. We used in-vivo double probe microdialysis to measure extracellular levels of histamine, and immunohistochemistry to detect ABT-239-induced c-Fos expression, in normal and acutely histamine-deprived rats (by means of a central injection of alpha-fluoromethylhistidine, an histamine biosynthesis inhibitor).

There is growing evidence for the procognitive effects of H3R antagonists/inverse agonists and the main hypothesis explaining the efficacy of these compounds is the regulation of the central cholinergic system. With these considerations in mind, in the second chapter we describe the results of experiments aimed at investigating the effects of ABT-239 and donepezil, an acetylcholinesterase inhibitor, in the object recognition test and evaluate the intracellular signaling pathways involved in these effects. Moreover we explored the requirement of an intact neuronal histaminergic system assessing the

behavioural and neurochemical effects of these compounds in acute and chronic histamine-deficient rodents.

In the third chapter we describe the results of experiments aimed at evaluating how manipulations of the central histaminergic system affect the anorexiant response and neurochemical modifications induced by oleoylethanolamide (OEA), an endogenous lipid-derived satiety messenger. Our hypothesis was that peripherally administered OEA engaged histamine signaling in the brain to fully exert it anorexiant effect. To reach this goal, we used a combination of behavioural, neurochemical and immunological techniques in normal and acutely or chronically histamine-deficient mice.

CHAPTER I

The antagonism of the histaminergic H3 receptor reveals heterogeneity of the histaminergic neurons in the rat Tuberomamillary Nucleus

Several studies demonstrated that somata of aminergic neurons are restricted to discrete cell clusters in distinct brain regions, although their axons innervate nearly the entire CNS. For instance, locus coeruleus, substantia nigra and dorsal raphe are sources of noradrenergic, dopaminergic and serotonergic axons, respectively. These nuclei are comprised of distinct compartments with respect to projection fields, as separate groups of noradrenergic (A1-A7), dopaminergic (A8-A17) and serotonergic (B1-B9) neurons give rise to distinct sets of axons innervating separate brain regions (Mason & Fibiger, 1979; Jacobs & Azmitia, 1992). This organization with nuclei innervated by the same group of monoaminergic neurons implies in the presence of sets of noradrenergic, dopaminergic or serotonergic neurons with independent functions according to their origin and terminal projections.

In a similar way, the tuberomamillary nucleus (TMN) of the posterior hypothalamus was identified as the sole source of neuronal histamine in the rodent brain (Panula *et al.*, 1984). These neurons are organized in five distinct (E1-E5) cell groups and give rise to extensive network of histidine decarboxylase- and histamine-immunoreactive fibres that innervate almost the entire forebrain and parts of the spinal cord (Ericson *et al.*, 1987; Inagaki *et al.*, 1988a). In opposition to other aminergic systems, retrograde tracers injected into different CNS regions labelled histaminergic cell bodies scattered throughout the TMN without a strict topographical pattern (Kohler *et al.*, 1985; Inagaki *et al.*, 1990). This observation suggested that histaminergic neurons are a homogeneous cell group acting as a regulatory network for whole brain activity through diffuse projections rather than processing specific functions (Wada et al., 1991). Recently, evidence has beginning to accumulate showing the heterogeneity of histaminergic neurons (reviewed in (Blandina *et al.*, 2012).

Heterogeneity of histaminergic neurons in response to stress

(Miklos & Kovacs, 2003) evaluated the effects of different types of stress (restrain, intermittent electric footshock, immobilization, dehydration, hypoglycaemia, acute hyperosmotic stimulus, ether inhalation, and bacterial lipopolysaccharide challenge) on histaminergic neuronal activation by using immunohistochemistry to measure c-fos expression, a marker of cell activation, in histamine neurons identified with *in situ* hybridization of histidine decarboxylase

(HDC) mRNA. Under stress-free, basal conditions, just a few, scattered c-Fos positive cell nuclei were detected in histamine neurons in the TMN. The remarkable observation is that there was a subgroup-specific activation of histaminergic neurons in response to stress since restrain, hypoglycaemia and shock activated E4 and E5 histamine neurons, but failed to induce any significant activation in the histamine neurons of the three remaining clusters. These results suggest that histaminergic neurons of distinct TMN clusters are recruited in a stressor- and subgroup-specific manner (Miklos & Kovacs, 2003).

In line with the concept of heterogeneity of histamine neurons, a briefly exposition of rats to hypercapnic gas conditions preferentially increased c-Fos expression in histaminergic neurons E2 cell group (Haxhiu *et al.*, 2001; Johnson *et al.*, 2005). Consistently, recordings in rat brain slices revealed that acidification within the physiological range (from pH = 7.4 to pH = 6.5) excited E2 neurons localized in the ventrolateral TMN. The excitation is mediated by both metabotropic glutamate receptors and acid sensing ion channels (ASICs) that are expressed at significantly higher density in the ventrolateral TMN than in the medial part (Yanovsky *et al.*, 2012)

Heterogeneity of histaminergic neurons by food-deprivation

Previous studies showed an increase on c-Fos expression in histaminergic neurons when hungry rats were presented to a mesh box with enticing food which they can smell and see but they can't eat (Valdes *et al.*, 2005; Valdes *et al.*, 2010). These studies, however did not described exactly what subdivisions of TMN became activated. This issue was addressed by Umehara *et al.*, 2011 using a food deprivation under scheduled feeding protocol. Rats were habituated to receive food at the same time and for a restrict period of the day (2h). After habituation, in the experimental day, hungry rats were presented with food, but when they started to eat, food was immediately removed. It was observed a great increase on c-Fos expression in the cells of the E3 subdivision whereas few cells were activated in the other subdivisions. These results are in accordance with the role of the histaminergic system in the modulation of motivated arousal. Although food-deprivation could be considered a kind of stress these studies demonstrated that it engages in the TMN different neuronal populations as compared with other types of stress like shock (Miklos & Kovacs, 2003; Umehara *et al.*, 2012)

Heterogeniety of histaminergic neurons in response to GABA and glycine

Electrophysiological studies using whole-cell recording identified two subpopulations among histamine neurons in the TMN according to their sensitivity to GABA: one population with maximal responses ranging between 0.5 and 5 μM and another one with similar responses between 50 and 500 μM (Sergeeva *et al.*, 2002). The GABA receptor complex is formed by the assemblage of different subunits (α, β and γ) forming hetero-oligomeric pentamers. According with the subunit composition this receptor shows differential sensitivity to GABA and other modulators like steroids, benzodiazepines, etc. (Olsen & Sieghart, 2009). Thus, the authors investigated the expression patterns of 12 $GABA_A$-R subunits single cell RT-PCR in histaminergic neurons and correlated with GABA EC_{50}. As conclusion, they found that TMN neurons expressing both gamma1 and gamma2 subunits displayed a high sensitivity to GABA, whereas the group expressing only the gamma2 subunit displayed a low sensitivity (Sergeeva *et al.*, 2002; Sergeeva *et al.*, 2005).

Histaminergic neurons are also heterogeneous with respect to their sensitivity to glycine which correlates with their size. Two groups of cell were identified: the frist, a group of histaminergic cells with larger somata (25 μm) had pronounced glycine responses (10-1000 μM) with a maximal current amplitude between 0.4 and 2.7 nA. The cells with a smaller soma size (19.5 μm) had negligible glycine responses or did not respond at all (Sergeeva *et al.*, 2001)

Histamine neurons establish funcionally distinct pathways according to their terminal projections

In our laboratory in the past years we addressed the question of whether histaminergic neurons are organized into functionally distinct circuits. We used the double-probe microdialysis technique in freely moving animals, a technique that provides a powerful means to study the dynamics regulating neurotransmitter release in discrete brain regions (Lee *et al.*, 2008). Rats were implanted with one probe in the TMN, to deliver drugs and measure histamine release locally, and another probe to measure histamine release from histaminergic projection areas such as the prefrontal cortex (pFCx), the nucleus basalis magnocellularis (NBM), the nucleus accumbens (NAcc) or the dorsal striatum (STR). By applying compounds targeting receptors expressed on histamine neurons and measuring

histamine output in different brain areas, we demonstrated that the same compound modified the release of histamine only from selected brain regions. The $GABA_A$-R antagonist, bicuculline acts directly onto histaminergic neurons to augment cell firing (Haas *et al.*, 2008). We found that intra-hypothalamic perfusion of bicuculline increased histamine release from the TMN, the NAcc and the pFCx, but not from the STR (Giannoni *et al.*, 2009). Different subunit composition and stoichiometry of $GABA_A$-Rs among histaminergic neurons (Sergeeva *et al.*, 2002; Sergeeva *et al.*, 2005) may account for these results.

Cannabinoid receptor 1 (CB1) agonists differentialy activate histaminergic neurons according to their projections. Administration of methanandamide (mAEA) or arachidonyl-2'-chloroethylamide (ACEA) in the TMN facilitated histamine release from the TMN itself, from the NBM and STR as well (Cenni *et al.*, 2006). However, perfusion of the posterior hypothalamus with mAEA did not change significantly histamine release from the perirhinal cortex (Passani *et al.*, 2007) despite the profuse histaminergic innervation of this region (Panula *et al.*, 1989).

We further demonstrated that histaminergic neurons are not a homogenous neuronal population by using H3R. When applied to the rat TMN, thioperamide or GSK-189254 invariably augmented histamine release from the TMN, from the pFCx and from the NBM. Despite the fact that the NAcc and the STR receive histaminergic innervations (Panula *et al.*, 1989), histamine levels remain stable in these regions after H3R antagonist perfusion into the TMN (Giannoni *et al.*, 2009; Giannoni *et al.*, 2010). Local perfusion with the same compounds in the NBM or in the pFCx augmented significantly histamine release within these regions, whereas locally perfusion into the STR or NAcc did not modify histamine release, thus indicating that the whole somatodendritic domain of histaminergic neurons projecting to these regions are insensitive to these drugs (Giannoni *et al.*, 2009; Giannoni *et al.*, 2010)

ABT-239: a potent and selective H3R antagonist/inverse agonist

In the present work we evaluated the effects of a non-imidazole H3R antagonist ABT-239 [4-(2-{2-[(2R)-2-Methylpyrrolidin-1-yl]ethyl}-benzofuran-5-yl)benzonitrile], that belongs to the family of ligands based on a 2-aminoethylbenzofuran skeleton synthetized by the Abbot laboratories. It shows high affinity for the human and rat H3Rs and is over 1000-fold more selective

versus human H1, H2, and H4 histamine receptors (Cowart *et al.*, 2005). ABT-239 is a potent H3R antagonist, reversing agonist-induced changes in cAMP formation, [^{35}S]GTPγS binding, calcium mobilization and competitively reversed histamine-mediated inhibition of [^{3}H]histamine release from rat brain cortical synaptosomes. Additionally, ABT-239 is a potent inverse agonist, inhibiting constitutive [^{35}S]GTPγS binding at both rat and human H3R (Esbenshade *et al.*, 2005).

In vivo, ABT-239 ameliorates attention deficits in the five-trial inhibitory avoidance test in spontaneous hypertensive rat pups, one of the most widely accepted animal models for attention deficit/hyperactivity disorder. Social memory was also improved in adult and aged ABT-239-treated rats. At higher doses ABT-239 partially reversed scopolamine-induced deficits in spatial memory in rats in the water maze (Fox *et al.*, 2005). Moreover, concomitant administration of ABT-239 and nicotine further improved memory acquisition and consolidation in mice as measured in the modified elevated plus maze used as a memory test (Kruk *et al.*, 2012). Alcohol consumption during rat pregnancy induces a learning-deficits in the adult offspring revealed as a worst performance in fear conditioning and water maze tests as well as an impairment in the LTP induced by 3 tetanizing trains into the dentate gyrus respect to control animals. ABT-239 treatment reverted aversive and spatial memory and LTP deficits induced by prenatal ethanol exposure (Savage *et al.*, 2010; Varaschin *et al.*, 2010) suggesting its usefulness for treating fetal ethanol-associated synaptic plasticity and learning deficits.

The treatment with ABT-239 improved also animal performance in models related to schizophrenia: decreased methamphetamine-induced hyperactivity in mice; attenuated the intrinsic deficits of pre-pulse inhibition in DBA/2 mice (Fox *et al.*, 2005), but did not revert the inhibitory effect induced by apomorphine injections in the same model (Burban *et al.*, 2010). ABT-239 reverted cognitive deficits induced by the N-methyl-d-aspartate (NMDA) receptor antagonists (ketamine and MK-801) on spontaneous alternation in cross-maze and inhibitory avoidance tests in rats and mice, respectively (Brown *et al.*, 2013). Furthermore, chronic ABT-239 treatment ameliorated working memory impairments observed in rats prenatally treated with methylazoxymethanol (methylating agent that disrupt neurogenesis) a neurodevelopmental model for schizophrenia (Brown *et al.*, 2013).

Although the central mechanisms mediating the behavioural effects of ABT-

239 are not completely understood, *in vivo* microdialysis studies showed that increases in cortical and hippocampal ACh release are in agreement with doses and time courses of its efficacy in behavioural and cognitive models (Fox *et al.*, 2005). An increase of dopamine levels in the pFCx, but not from the STR, was also observed after ABT-239 treatment (Fox *et al.*, 2005). The modulation of dopamine release in selected brain regions may also be of therapeutic relevance in the treatment of schizophrenia, as insufficient dopamine D1R activation in prefrontal cortex has been reported as a potential cause of the cognitive deficits in schizophrenia. Moreover, its lack of effects on striatal dopamine release suggests that ABT-239 would not induce extrapyramidal side effects reviewed in (Passani & Blandina, 2011; Passani *et al.*, 2011).

ABT-239 shows also a very interesting pharmacokinetic profile: it exhibited high volumes of distribution in combination with low clearance values, providing elimination half-lives averaging 5.3, 8.3, and 29.2 hours in rat, dog, and monkey, respectively. Oral bioavailability ranges between 52% and 89% among the studied species. ABT-239 shows also blood-brain-barrier penetration as demonstrated by levels that were 21.6- and 15.8-fold higher in the rat brain than levels in the blood at 1 and 5 hours after dosing (Esbenshade *et al.*, 2005).

Subsequent cardiovascular safety studies in monkeys unfortunately precluded future ABT-239 clinical studies. Although no cardiovascular events were noted at oral doses of 0.1 and 0.3 mg/kg, oral doses of 1 mg/kg, expected to produce peak blood levels of about 158 ng/mL, were associated with QT prolongation in all animals. Since the efficacious exposure levels in rat behavioral models ranged from 0.22 to 4.7 ng/mL, the estimate margin of safety (158/4.7) was only 30-fold, which was deemed insufficient for clinical evaluation (Hancock, 2006). However, due to its potency and selectivity for the H3R, it is an excellent tool compound to investigate the role of H3R in the brain.

AIMS

In the present study we aimed to further investigate the differential sensitivity of histaminergic neurons to H3R antagonists. We studied the neurochemical effects of intra-TMN perfusion with ABT-239 on neuronal activation of histaminergic projection areas using single and double-probe microdialysis and

immunohistochemical techniques. Moreover the requirement of an intact histaminergic transmission for the ABT-239 effects was evaluated in animals treated with alpha-fluoromethylhistidine (αFMH) an irreversible inhibitor of the enzyme responsible for the histamine biosynthesis.

METHODS

Animals

Male Sprague Dawley rats (225-275 g) from the Harlan Laboratories (Milan, Italy) were used. Animals were housed at the Centro Sabulazione Animali di Laboratorio (CeSAL). Università di Firenze in groups of three in a temperature-controlled room (22-24 °C), allowed free access to food (Teklad Global 18% protein rodent diet) and water, and kept on a 12-h light/dark cycle (light starts at 7:00 AM). Alternatives to *in vivo* techniques were not available, but all efforts were made to minimize animal suffering and to reduce the number of animals used. All the experiments were complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC) recommendations for the care and use of laboratory animals, and local ethical review.

Compounds

ABT-239 and α-fluoromethylhistidine (αFMH) were synthesized at Abbott Laboratories (Abbott Park, IL 60064, USA). All other reagents and solvents were of HPLC grade or the highest grade available (Sigma, UK).

Microdialysis in freely moving rats.

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and positioned in a stereotaxic frame (Stellar, Stoelting Co., Wood Dale, IL). One or two guide cannulae (MAB 4.15.IC, Microbiotech, Sweden) were implanted with according to the following coordinates from bregma (Paxinos & Watson, 1988): tuberomamillary nucleus (TMN) AP = -4.3, L = -1.1, DV = +7.2; nucleus basalis magnocellularis (NBM), AP = -0.8; L= - 2.8; DV = +6.5; dorsal striatum (STR), AP = 0, L = -4.0 DV = +4.0; nucleus accumbens (NAcc), AP = +1.7, L = -1.4, DV = +6.3; prefrontal cortex (pFCx), AP = +3.2; L = +1.0; DV = +2.8. A surgical screw served as an anchor and the cannulae were fixed to the skull with acrylic dental cement. The animals were then housed one by cage and left to recovery. The

microdialysis experiments were performed 48 h after surgery when the stylet was removed from the guide cannulae, and the microdialysis probes (2 mm, molecular weight cut-off = 6000 Da, MAB 4.15.2.PES Microbiotech, Sweden) were inserted. Probes were perfused with Ringer's solution (in mM: NaCl, 147; $CaCl_2$,1.2 and KCl, 4.0 at pH = 7.0) at a flow rate of 2 μl/min using a microperfusion pump (Carnegie Medicine, Sweden; Mod CMA/100). After 120 min equilibration, the sample collection started, at 15 min intervals. Spontaneous release was defined as the average value of the five 15-min fractions collected during 75 min of perfusion with Ringer's solution prior to drug treatment. All subsequent fractions were expressed as percentage of this value.

In the dual-probe experiments, both probes were perfused with control medium in the first five 15-min samples to measure histamine spontaneous release; ABT-239 (10 μM final concentration) was then added only to the TMN-perfusing medium, dissolved in the Ringer's solution (drug addiction did not modify the pH of the medium). In the single-probe experiments the brain region of interest was perfused with control medium in the first five samples, and ABT-239 was then added to the perfusing medium (Figure I.1).

To prevent degradation of histamine, 1.5 ml of 5 mM HCl was added to each sample. The dialysates were kept at -80 °C until analysis.

Histology

The placement of microdialysis membranes was verified post-mortem. Rats were overdosed with chloral hydrate, the brains removed and stored in 10% formalin for 10 days. Forty μm sections were sliced on a cryostat, mounted on gelatine-coated slides and then stained with cresyl violet for light microscopic observation. Data from rats in which the membranes were not correctly positioned were discarded (less than 5%).

Determination of histamine

Histamine contents in the dialysates were determined by HPLC-fluorimetry after post-column derivatization as described previously (Giannoni *et al.*, 2010). The HPLC system used to quantify histamine consisted of an auto sampler, a micro vacuum degasser, an oven, four isocratic pumps and a fluorimetric

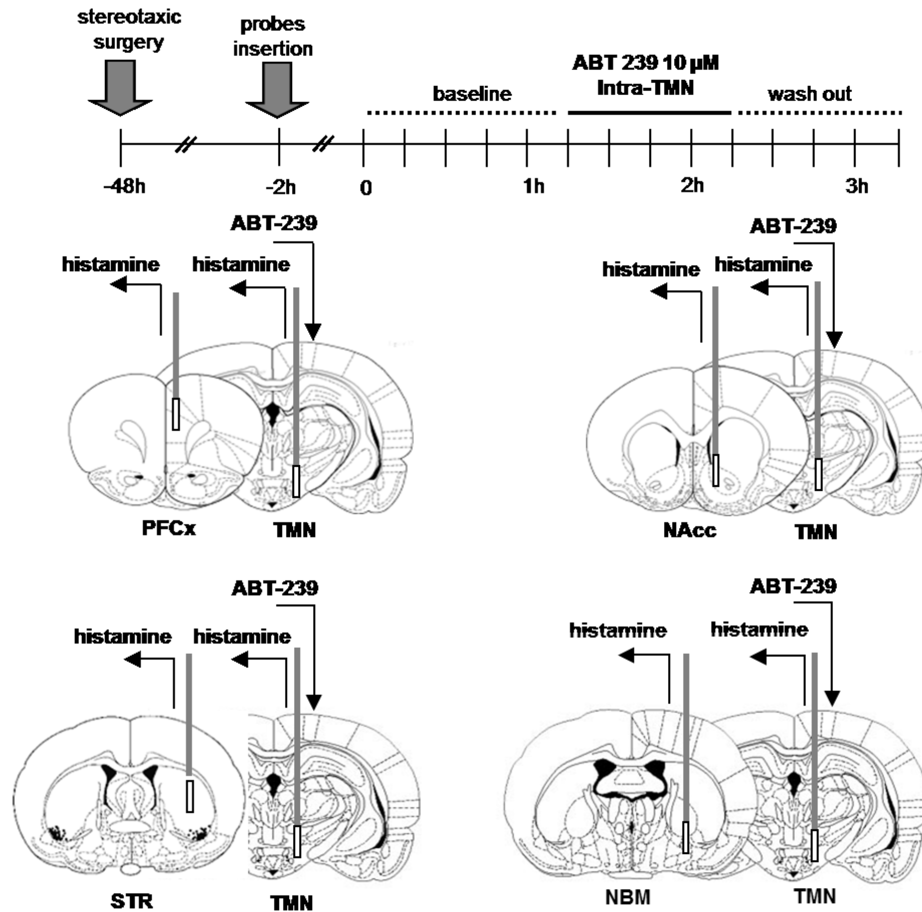

Figure I.1. Schematic diagram showing experimental protocol and the position of the microdialysis probes. Rats were implanted with two ipsilateral probes: one probe in the Tuberomamillary nucleus (TMN) to deliver ABT-239 (10 µM) locally and measure neurotransmitter release, and a second probe in an histaminergic projection area: the Prefrontal Cortex (pFCx), the Nucleus Accumbens (NAcc), the Dorsal Striatum (STR) or the Nucleus Basalis Magnocellularis (NBM) to measure histamine release.

detector (all devices Agilent 1100 series, Agilent, USA). The temperature of the rack containing the samples was set at 4 °C. Briefly, the column (Poroshell 120 EC-C18, 2.1 x 75mm, 2.7µm, Agilent, USA) was eluted with 0.25 M potassium dihydrogen phosphate containing 5% octanesulfonic acid at a flow rate of 0.3 mL/min. The eluate from the column was mixed first with 0.1% *o*-phthalaldehyde solution at a flow rate of 0.1 mL/min and then to a solution containing 4M sodium hydroxide and 0.2M boric acid (flow rate 0.137 mL/min) to adjust the reaction mixture to pH = 12.5. The reaction took place at 45 °C. Then 17% orthophosphoric acid was added to the solution (flow rate 0.137 mL/min) to reach a final reaction mixture at pH = 3.0. The fluorescent intensity was measured with a spectrofluorometer at 450 nm with excitation at 360 nm. The sensitivity limit was 10 fmol and the signal/noise ratio was higher than 3. Histamine levels in the

dialysate samples were calculated as fmol/15 min, and were not corrected for probe recovery (about 40%).

Immunohistochemistry.

c-Fos immunohistochemistry was used as an indicator of *in vivo* neuronal activation using the methodology previously described (Bacciottini *et al.*, 2002). Briefly, the TMN of rats was perfused for 60 min through a microdialysis probe with plain Ringer's solution or Ringer's solution containing ABT-239 (10 μM final concentration), at a flow rate of 2 µl/min using a microperfusion pump. Ninety min after the end of perfusion, rats were deeply anaesthetized and perfused transcardially with ice-cold 4% paraformaldehyde. In another set of experiments, animals received 5 μg of αFMH into the lateral ventricle (AP = -0.9, L = -1.5, DV = +3.6) during the stereotaxic surgery and 48 h after they were submitted to the same experimental protocol described above. The brains were removed and post-fixed in 4% paraformaldehyde overnight and then immersed in 30% sucrose for 72 h. Coronal sections were cut at 40 μm on a freezing cryostat, and were collected in phosphate buffer (PB) and stored at -20 °C until processed for immunohistochemistry. Free-floating sections were incubated with anti c-Fos polyclonal antibodies (1:5000, Sigma-Aldrich) diluted in PB containing 0.3% Triton X-100 and 5 mg/mL bovine serum albumin (BSA), overnight. The immunoreactive product was detected with the avidin-biotin peroxidase system (Vectastain kit; Vector Laboratories, Burlingame CA, USA). After washing with PB, sections were mounted on gelatine-coated slides, dehydrated in increasing concentrations of EtOH and coverslipped. Sections were observed using an Olympus BX40 microscope equipped with a Nikon DS-F1 camera. Atlas coordinates (AP, relative to the bregma) for the sections analysed were from +3.2 to +2.7 for the pFCx, from +1.7 to +1.2 for the NAcc, from +0.5 to 0.0 for the STR and from -0.4 to -0.6 for the NBM (Paxinos & Watson, 1988). The number of c-Fos immunopositive nuclei was counted using the Image J software (NIH, USA) on three to six sections per region for each rat. The number of positive nuclei was normalized to an area of 1 $mm^{2.}$ Statistics were calculated on the average values from three to six sections of individual animals. Thus, sample size and statistics were based on number of rats.

Statistical analysis

All values are expressed as means ± SEM, and the number of animals used in each experiment is indicated in the respective figure legends. The presence of significant treatment effects was first determined by a one-way ANOVA followed by Bonferroni test or Newman-Keuls test. For all statistical tests, $P<0.05$ was considered significant. For clarity, we reported in figures and figure legends of microdialysis experiments only the significant differences vs. the last sample before drug treatment. However, differences were significant vs. all baseline samples. Statistical analysis was performed using StatView (Abacus Concepts, Inc. Berkley, CA, USA), GraphPad Prism (GraphPad Software, Inc. La Jolla, CA, USA).

RESULTS

Microdialysis experiments

Rats were housed one by cage 48 hours before the experiments which were performed in a quiet room. c-Fos experiments were performed under the same conditions. Histological analysis confirmed that the probes were located in the correct areas, with no signs of unusual tissue damage or bleeding. Electrophysiological recordings of histamine neurons in freely moving animals indicate that their activity is high during waking and attention, and low or absent during sleep (Takahashi *et al.*, 2006). In the present study, since the experiments were performed during the light phase, histamine cells activity was likely low. Consistently, about 90% of the rats were sleeping before ABT-239 administration that produced clear signs of awakening (eating, drinking, grooming, moving in the cage), but not of agitation or irritability, as described also in (Giannoni *et al.*, 2009). After 120 min of equilibration following the insertion of the dialysing membranes, histamine was released spontaneously at a stable rate from all brain regions investigated.

Effects of TMN perfusion with ABT-239 on histamine release from the TMN and the pFCx

Using a double-probe microdialysis protocol, histamine release was monitored from both the TMN and the pFCx. After collection of five, 15-min baseline samples, ABT-239 (10 μM) was infused for 60 min locally into the TMN,

where all histaminergic neuronal cell bodies are localized, while the pFCx, that receives histaminergic innervation (Panula *et al.*, 1989) was always perfused with control medium. Infusion of ABT-239 elicited a significant increase of histamine release from both the TMN (ANOVA, $F_{(12,58)}$ = 7.743, P<0.0001) and the pFCx (ANOVA, $F_{(12,60)}$ = 8.351, P<0.0001). Histamine release was restored to control levels during subsequent TMN perfusion with control medium (Figure I.2). In the TMN the mean spontaneous release of histamine was 52 ± 4 fmol/15 min, and the maximal increase produced by ABT-239 was 86 ± 10% (n = 6). In the posterior hypothalamus histamine was likely released by short projections, as histaminergic neurons display extensive axonal arborisations within this brain region (Inagaki *et al.*, 1988a). In the pFCx, where histamine was released from TMN projections, the mean spontaneous release was 47 ± 7 fmol/15 min, and the maximal increase 95 ± 33% (n = 6).

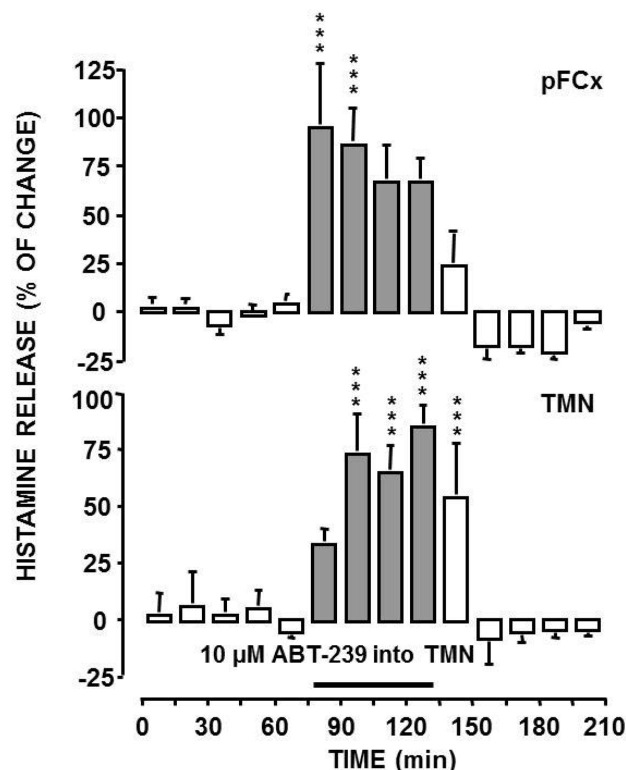

Figure I.2. Influence of ABT-239 (10 µM) perfusion into the TMN on histamine release from the TMN (lower panel) and the prefrontal cortex (upper panel) of freely moving rats. Histamine was measured in fractions collected every 15 min. Control values of spontaneous neurotransmitter release were calculated for each experiment by averaging the mean of five initially collected 15-min samples and histamine release was expressed as a percentage of calculated spontaneous release. The mean spontaneous release of histamine was 52 ± 4 fmol/15 min in the TMN, and 47 ± 7 fmol/15 min in the pFCX. Bar indicates the period of drug application. Shown are means ± SEM of 6 rats. ***P<0.001 vs. last sample before drug treatment (ANOVA and Bonferroni test).

Effects of TMN perfusion with ABT-239 on histamine release from the TMN and NBM.

ABT-239 induced a significant increase of histamine release from both the TMN (ANOVA, $F_{(12,26)}$ = 9.429, P<0.001) and the NBM (ANOVA, $F_{(12,26)}$ = 7.458, P<0.001), which returned to baseline values after perfusion with ABT-239 ended. In the TMN the maximal increase was 105 ± 10% (Figure I.3). When ABT-239 was infused into the TMN, histamine release from TMN projections into the NBM increased up to a maximum of 107 ± 27% (Figure I.3). Histamine spontaneous release from the TMN averaged 40 ± 4 fmol/15 min, and from the NBM averaged 44 ± 5 fmol/15 min (n = 3).

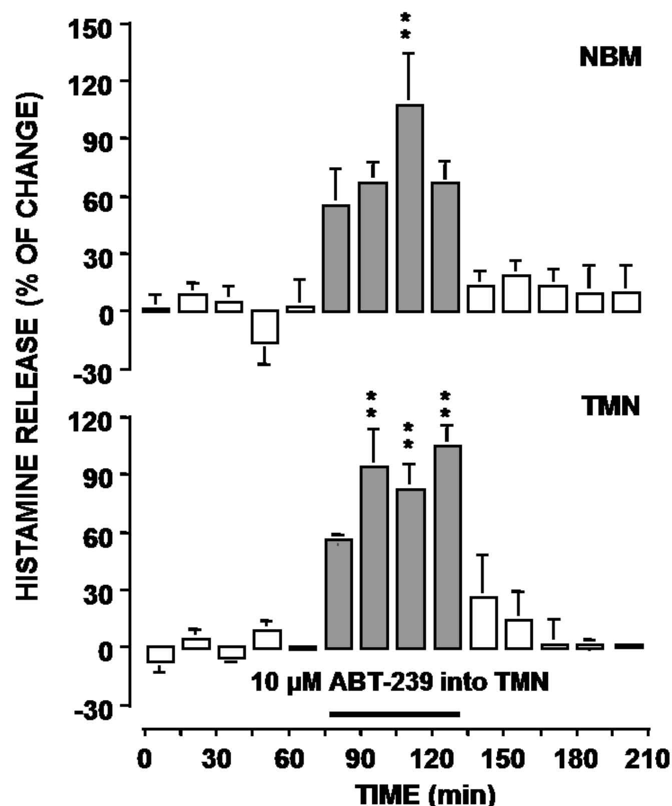

Figure I.3. Influence of ABT-239 (10 μM) perfusion into the TMN on histamine release from the TMN (lower panel) and the nucleus basalis magnocellularis (upper panel) of freely moving rats. Histamine was measured and calculated as described in Figure I.2. The mean spontaneous release of histamine was 44 ± 5 fmol/15 min in the TMN, and 40 ± 4 fmol/15 min in the NBM. Bar indicates the period of drug application. Shown are means ± SEM of 3 rats. **P<0.01 vs. last sample before drug treatment (ANOVA and Bonferroni test).

Effects of TMN perfusion with ABT-239 on histamine release from the TMN and the NAcc.

When ABT-239 (10 μM) was added to the TMN-perfusing medium for 60

min, the spontaneous release of histamine from the TMN increased significantly by a maximum of 114 ± 18% (ANOVA, $F_{(12,61)}$ = 13.163, P<0.001), but was not significantly changed in the NAcc, since the changes remained within variability range (approx. 20%) observed between individual 15-min collection periods during perfusion with control medium (Figure I.4). The mean spontaneous release of histamine was 41 ± 4 fmol/15 min in the TMN, and 33 ± 3 fmol/15 min in the NAcc (n = 6).

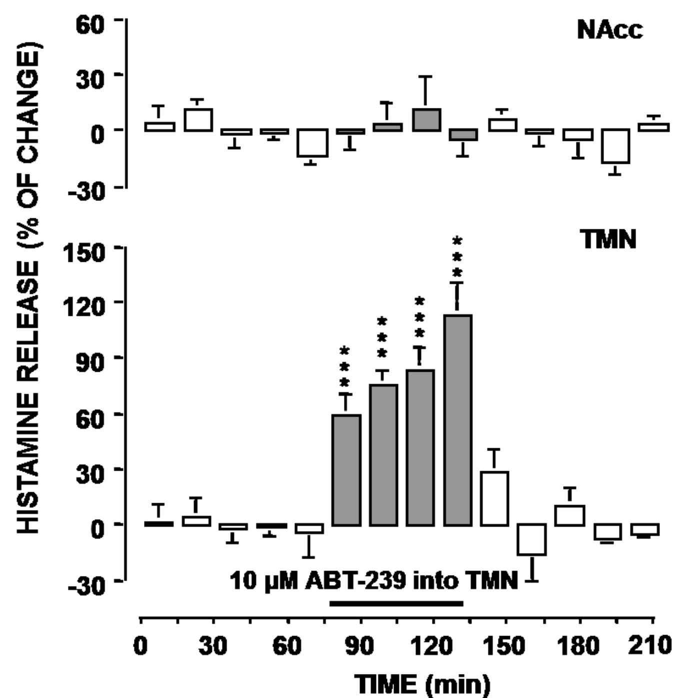

Figure I.4. Influence of ABT-239 (10 µM) perfusion into the TMN on histamine release from the TMN (lower panel) and the Nucleus accumbens (upper panel) of freely moving rats. Histamine was measured and calculated as described in Figure I.2. The mean spontaneous release of histamine was 41 ± 4 fmol/15 min in the TMN, and 32 ± 3 fmol/15 min in the NAcc. Bar indicates the period of drug application. Shown are means ± SEM of 6 rats. ***P<0.001 vs. last sample before drug treatment (ANOVA and Bonferroni test).

Effects of TMN perfusion with ABT-239 on histamine release from the TMN and the STR.

Perfusion of the TMN with 10 µM ABT-239 increased significantly histamine release from the TMN (ANOVA, $F_{(12,25)}$ = 4.328, P<0.001) but not from the striatum. Mean histamine spontaneous release from the TMN was 51 ± 1 fmol/15 min and ABT-239 elicited a maximal increase of 124 ± 25% (Figure I.5). Histamine levels returned to basal values during washout of the compound. Conversely, no significant change was observed in the release of histamine from the dorsal

striatum, the changes being within variability range (approx. 20%) observed between individual 15-min collection periods during perfusion with control medium (Figure I.5). Histamine spontaneous release from the dorsal striatum was 56 ± 2 fmol/15 min (n = 3)

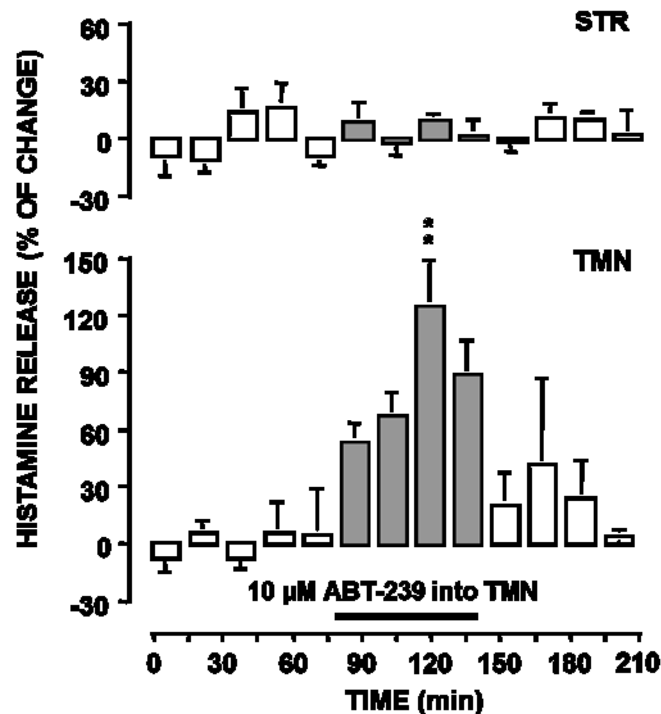

Figure I.5. Influence of ABT-239 (10 μM) perfusion into the TMN on histamine release from the TMN (lower panel) and the dorsal striatum (upper panel) of freely moving rats. Histamine was measured and calculated as described in Figure I.2. The mean spontaneous release of histamine was 56 ± 2 fmol/15 min in the TMN, and 51 ± 1 fmol/15 min in the NBM. Bar indicates the period of drug application. Shown are means ± SEM of 3 rats. **P<0.01 vs. last sample before drug treatment (ANOVA and Bonferroni test).

Single-probe experiments: effects of local perfusion with ABT-239 on histamine release from the NBM and NAcc.

To learn the effects of ABT-239 at the axonal domains of histaminergic neurons, each rat was implanted with a single probe, in the NBM or the NAcc, to administer locally ABT-239 and monitor changes in histamine release. NBM perfusion with 10 μM ABT-239 for 60 min elicited a significant, transient increase of histamine release (ANOVA, $F_{(11,48)}$ = 10.52, P<0.0001) with a maximal value of 111 ± 15% (Figure I.6). Increased histamine levels persisted during perfusion with ABT-239, after which basal histamine levels were quickly attained. The mean spontaneous release of histamine from the NBM was 59 ± 8 fmol/15 min (n = 5).

Perfusion of the NAcc with 10 μM ABT-239 for 60 min failed to produce any significant change in the spontaneous release of histamine (Figure I.6). The mean spontaneous release of histamine from the NAcc was 37 ± 5 fmol/15 min (n = 6).

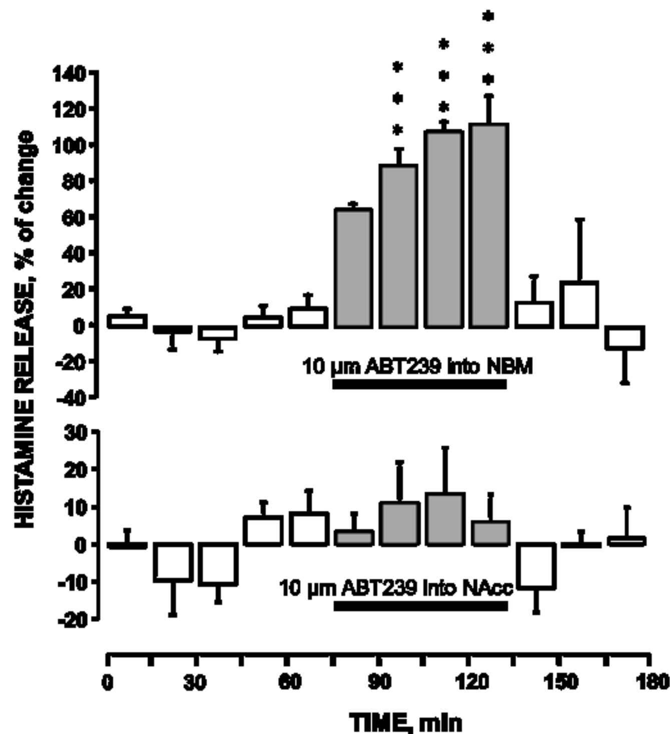

Figure I.6. Effects of local perfusion with ABT-239 on histamine release from the NBM (upper panel), and the NAcc (lower panel) of freely moving rats: single-probe experiments. Each rat was implanted in the NBM or NAcc with a probe used to simultaneously administer ABT-239 locally and monitor changes in histamine release. Histamine was measured and calculated as described in Figure I.2. The spontaneous release of histamine averaged 59 ± 8 fmol/15 min (n = 5) from the NBM, and 37 ± 5 fmol/15 min (n = 6) from the NAcc. Both regions were perfused with 10 μM ABT-239 for 60 min. Bars indicate the period of ABT-239 application. Shown are means ± SEM of 5 (A), and 6 (B) rats. ***P<0.001 vs. last sample before drug treatment (ANOVA and Bonferroni/Dunn test).

Intra-TMN administration of ABT-239 increased c-Fos expression in selected brain regions.

Compared with vehicle-treated animals the density of c-Fos staining was increased in rat brain following the perfusion of TMN through a microdialysis probe for 60 min with a solution containing 10 μM ABT-239. Quantification of this effect showed that ABT-239 induced statistically significant increases in c-Fos immunoreactive nuclei in the pFCx (ANOVA $F_{(2.8)}$=5.846, P<0.05) and the NBM (ANOVA $F_{(2.8)}$= 9.810, P<0.05), but not in the STR nor in the NAcc (unpaired t-test, P>0.05) (Figure I.7). The pre-treatment with αFMH (5 μg, i.c.v), an irreversible

inhibitor of histidine decarboxilase, prevented the increase of c-Fos immunoreactive nuclei observed in both the pFCx and the NBM after 60 min perfusion of ABT-239 10 μM into the TMN (Figure I.7).

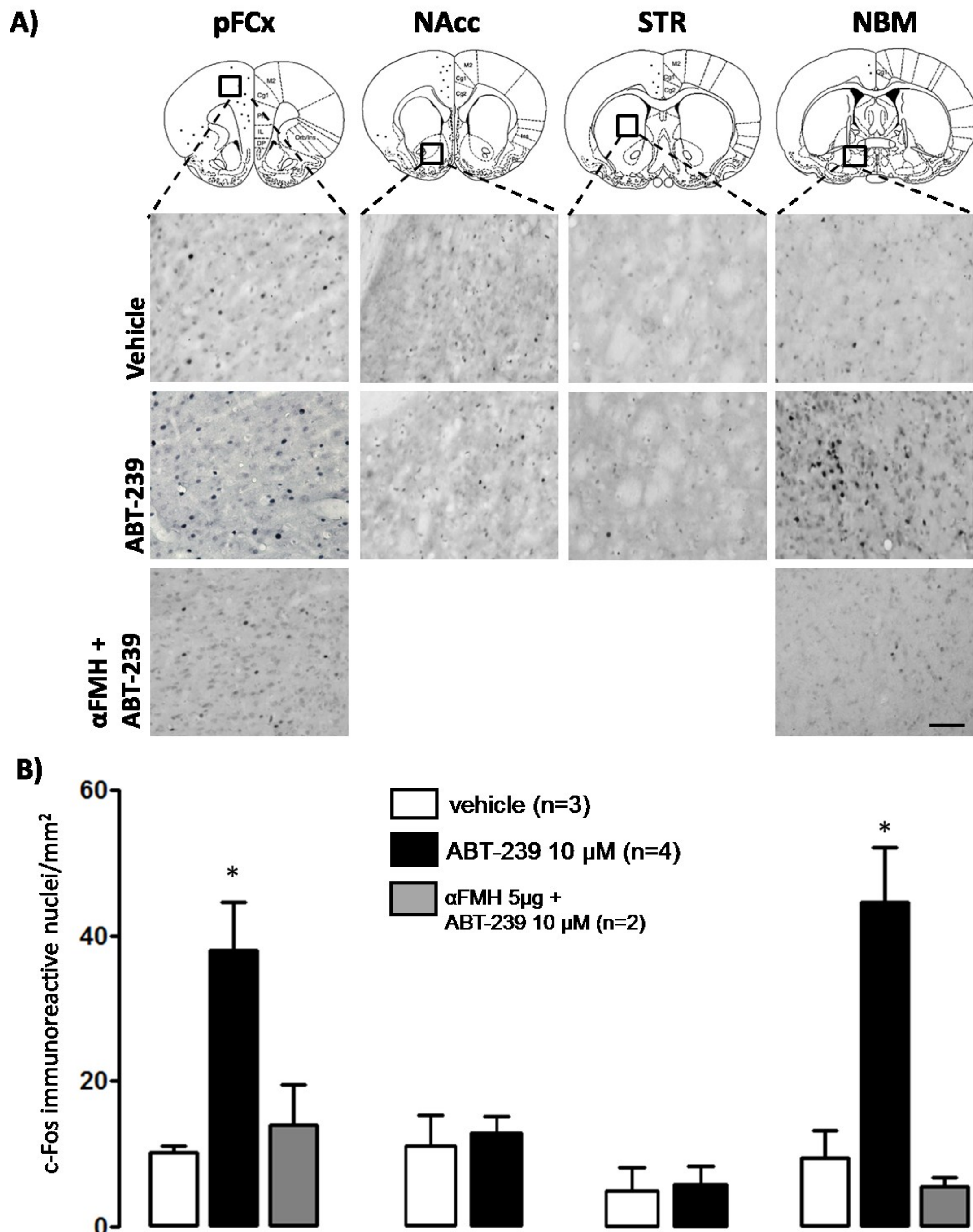

Figure I.7. Influence of ABT-239 administration into the TMN on c-Fos expression in selected brain regions of normal and histamine-deprived rats. (A) Representative photomicrographs illustrating c-Fos immunostaining in the NBM, the pFCx, the STR and the NAcc following TMN perfusion with either vehicle or ABT-239 (10 μM) for 60 min. aFMH (5μg) was injected in the lateral ventricle during the stereotaxic surgery. Scale bar, 100 μm. (B). Effects of ABT-239 (black bars) on the density (counts per square millimeter) of c-Fos immunopositive nuclei in animals treated with vehicle (white bars), ABT-239 (black bars) or both aFMH and ABT-239 (gray bars). Shown are means ± SEM from 2-4 rats by experimental group. *P<0.05 versus correspondent vehicle-treated animals, ANOVA and Newman Keuls test.

DISCUSSION

Histamine axons originate from a single source, the TMN in the posterior hypothalamus, to innervate almost all CNS regions (Haas *et al.*, 2008), and the histamine system was generally regarded as one single functional unit that provided histamine throughout the brain (Wada *et al.*, 1991). However, growing evidence suggest diverse physiological roles served by different histamine neuronal subpopulation (Blandina *et al.*, 2012). Since microdialysis provides a powerful means for defining the dynamics regulating histamine release in discrete brain regions, the present study, using the double-probe microdialysis technique in freely moving animals, addressed the question whether histaminergic neurons are organized into distinct functional circuits impinging on different brain regions. Rats were implanted with one probe in the TMN, to deliver ABT-239 and measure histamine release locally, and another probe to measure histamine release from histaminergic projection areas. ABT-239, applied locally into the TMN, significantly increased histamine release from the TMN, the pFCx and the NBM, but not from the STR, nor the NAcc. The present findings complement earlier observations that thioperamide and GSK189254, an imidazole and a non-imidazole H3R antagonist, respectively, if applied to the rat TMN invariably augmented histamine release from the TMN, the pFCx the NBM, but not from the NAcc, nor the STR (Giannoni *et al.*, 2009; Giannoni *et al.*, 2010). Taken together these results strongly support the hypothesis that H3R antagonists, as a class of agents, differentiate histaminergic neurons according to their projection areas.

The H3R is present on presynaptic terminals and varicosities of histaminergic axons, where it moderates histamine synthesis and release (Arrang *et al.*, 1983). H3Rs are also located on histaminergic neuronal somata, and provide a tonic inhibition (Haas *et al.*, 2008) by modulating inward Ca^{2+} currents (Reiner & McGeer, 1987; Takeshita *et al.*, 1998; Stevens *et al.*, 2001). Therefore, blockade of somatic and presynaptic H3-autoreceptors increases cell firing (Haas & Panula, 2003) and augments histamine levels in the synaptic cleft. In the TMN, histamine is released from short projections that form extensive axonal arborizations in the posterior hypothalamus. In the TMN, ABT-239 increased local histamine release likely as a consequence of both somatic and presynaptic H3-autoreceptors blockade.

Histamine increases in the prefrontal cortex and NBM during TMN perfusion

with ABT-239 were likely consequent of discharge potentiation of histamine neurons sending efferents to these regions, whereas the lack of increase in the STR and the NAcc, despite the fact that these brain areas receive histaminergic innervation (Inagaki *et al.*, 1988a; Panula *et al.*, 1989), indicate that histaminergic neurons projecting to these regions are insensitive to H3R blockade. The lack of response in the NAcc and the STR cannot be explained by spatial segregation due to probe localization, as retrograde tracing with dye injections into the striatum or prefrontal cortex showed that most histaminergic somata are within the medial part of the ventral TMN (Kohler *et al.*, 1985). This proximity suggested that histaminergic somata projecting to the STR and pFCx had the same exposure to H3R antagonists, but were not affected in the same way. Two histaminergic neuronal populations that differed significantly for H3R expression levels were described (Blandina *et al.*, 2012). Since the magnitude of neuronal responses to extracellular signals may depend on membrane receptor density, histamine neurons displaying low levels of the H3R are possibly those innervating the NAcc or STR. Alternatively, as several isoforms of the H3R displaying strong pharmacological differences have been described (Bongers *et al.*, 2007a), *in vivo* insensitivity to H3R antagonists may depend on high expression of particular isoforms. Interestingly, local perfusion of the NBM with ABT-239 increased significantly the release of histamine locally, thus suggesting the presence of H3-autoreceptors. Conversely, local perfusion of the NAcc with ABT-239 did not alter spontaneous histamine release, thus indicating that the entire somatodendritic domain of histaminergic neurons projecting to this region was insensitive to H3R blockade.

To gather more insight on the selective activation elicited by ABT-239, we examined c-Fos expression in rat brain regions after ABT-239 (10 μM) intra-TMN perfusion. In keeping with the microdialysis data, ABT-239 perfusion induced an increase in c-Fos immunoreactive cells in restricted rat brain regions such as the pFCx and the NBM, but not the STR nor the NAcc. The same c-Fos activation pattern was induced by systemic treatment with other H3R antagonists, JNJ-10181457 (Bonaventure *et al.*, 2007) and GSK189254 (Medhurst *et al.*, 2007). The pre-treatment with αFMH, an irreversible blocker of the enzyme responsible for histamine biosynthesis, prevented the increase in c-Fos expression observed in the pFCx and NBM after perfusion with ABT-239 locally into the TMN, suggesting

that the increased neuronal activation observed in the histaminergic projection areas are dependent of the histamine release. Further experiments are required to identify the neuronal population(s) expressing c-Fos, but their activation are possibly related to the selective increases of cortical cholinergic and/or histaminergic tone produced by ABT-239 at the same dose (Fox *et al.*, 2005). Both NBM and pFCx are acetylcholine rich regions and are important to elaborate cognitive responses. Therefore the increase of histamine release and the neuronal activation observed after ABT-239 perfusion suggest that these regions are involved in the procognitive effects elicited by ABT-239.

CONCLUSIONS

Histamine neurons originate from a single source in the posterior hypothalamus and have a major role in maintenance of arousal and contribute to modulation of appetite, energy homeostasis, cognition, etc. Here we confirm that H3R antagonists, are effective at modulating histamine release selectively on subpopulations of histaminergic neurons that project to specific brain regions, such as the pFCX and NBM. Presumably, augmented histamine release is responsible for the increased neuronal activation in these brain regions that are involved in cognitive functions regulated by histamine (Giovannini *et al.*, 1999; Cecchi *et al.*, 2001). On the contrary, no changes were observed in histamine release and neuronal activation in the STR, nor in the NAcc, following ABT-239 perfusion in the TMN suggesting that the pharmacological effects of ABT-239 may not induce extrapyramidal side effects or affect the reward systems. These findings clearly strengthen the hypothesis that histaminergic neurons are not a homogenous neuronal population. This observation could be relevant for the development of specific compounds that affect only subsets of histamine cells, thus increasing the target specificity.

CHAPTER II

ABT-239 and donepezil require the integrity of the central histaminergic system to exert its behavioural and neurochemical effects

Over a century ago, Dr. William James, an American philosopher and psychologist, asked a critical question that continues to guide current memory research: “Of some [experiences] no memory survives the instance of their passage. Of others, it is confined to a few moments, hours or days. Others, again, leave vestiges that are indestructible, and by means of which they may be recalled as long as life endures. How can we explain these differences?” (James, 1890 *apud* Roozendaal & McGaugh, 2011). More than one hundred years later several theories and discoveries about the mnemonic processes are available, but we are still trying to understand the mechanisms and to answer the question proposed by Dr. James.

The initial step in the formation of a new memory is the acquisition of information related to a particular trace, coming from external stimuli (sensory input arising from subject-environment interaction) or from internal representations (cognition and emotion). After the acquisition, information must be stored either for a brief or a long period of time. While that information remains stored, it can be accessed by the process known as retrieval. Memory, thus, comprises the processes responsible for the acquisition, retention and retrieval of a learned experience (Milner, 1998; Izquierdo *et al.*, 1999). Short-term memory retention (defined in minutes or hours) may be converted to long-term retention (defined in days, weeks, or even years) by a specific sequence of events called consolidation which starts immediately after the acquisition phase (Izquierdo *et al.*, 1999; McGaugh, 2000). Finally, as time passes, even the most consolidated memories may disappear, a process called forgetting.

Not all information reaching the central nervous system (CNS) is stored. Most inputs do not pass the acquisition phase, because they are filtered out by attentional and emotional mechanisms (Cahill & McGaugh, 1998). Only a few inputs selected for acquisition and retention are consolidated in long-term memories, and many of these are even forgotten. Only memories relevant for cognition, emotionally salient, focused by attention or associated to stronger sensory input will actually persist. In this context, forgetting may also be seen as a filter, since it only affects memories previously selected to persist by emotional and attentional filters. Thus, the brain systems responsible for learning and and storing memories have a mechanism for preventing information overload (Izquierdo *et al.*, 2006).

Memories can be modulated by experiences occurring about the time when it is learned, consolidated or retrieved (Cahill & McGaugh, 1998; Kandel & Squire, 2000). Several brain areas and neurotransmittorial systems are involved in the different phases of the mnemonic process, depending on the type of memory consolidated.

Some paradigms to evaluate learning and memory in animals

Declarative memories are type of memory we use to answers the questions like: “where?” “who?” “when?” Although this type of memory is conserved among many species, their external expressions are very different (Paul *et al.*, 2009). Humans, for example, can use language and other symbols to declare its. Animals, on the other hand, cannot represent such knowledge verbally or symbolically. Therefore, researchers need to find another way to recognize acquisition of a mnemonic trace in animal models. Although it is very difficult to build a perfect animal model they were widely used to elucidate the cellular and molecular mechanisms underlying learning and memory, and have played an important role in understanding human cognition (Razafsha *et al.*, 2013).

Pavlov in 1927 studying the conditioned response in dogs described for the first time an associative learning. Adaptations of the model were done in several species, but the most used to investigate memory processes are rodents, evaluated in the so called “fear conditioning”. In this paradigm the animal is placed in an new environment (context) were it receives a mild aversive stimulus such as a foot shock (unconditioned stimulus) associated with another stimulus such as a tone or a light (conditioned stimulus), that usually does not elicit a response. At the end of the trial the animal learns the association and the presentation of the conditioned stimulus elicits characteristic fear behaviour, called freezing (absence of movements). Promnesic agents are expected to increase, whereas amnesic manipulations to reduce, the freezing behaviour (Wehner & Radcliffe, 2004; Curzon *et al.*, 2009). Evidence suggests that the basolateral amygdala complex is critically implicated in acquisition, consolidation, retrieval and extinction of the cue-induced fear conditioning (Maren & Quirk, 2004). The prefrontal cortex seems to be important for fear consolidation and extinction (Sotres-Bayon & Quirk, 2010), whereas the hippocampus seems to be involved in the contextual modulation of fear conditioning and extinction (Maren *et al.*, 2013).

In another paradigm, the avoidance learning, the subject learn a response in order to avoid an aversive stimulus. Although variations of the passive avoidance task exist, typically the test consists of a two compartment apparatus differing in brightness, temperature, or height. The animal is placed on the ‘‘safe’’ side, which has some features not desirable for rodents (e.g., bright light or cool draft), and upon crossing the threshold to the other chamber, an electric footshock is presented through a grid floor. When re-tested, an increase in the latency to enter the “unsafe” compartment is related as a measure of learning (Bryan *et al.*, 2009). In a variation called inhibitory avoidance, the animal is positioned on a platform (safe) placed up an electrified grid floor. When the animal steps down the platform and touch with the four paws the grid floor it receives a mild foot shock. In analogy with the passive avoidance, if the mnemonic trace is consolidated, in the recall session, the animal will avoid the grid floor and spend more time in the platform (Izquierdo & McGaugh, 2000). Multiple systems and brain areas such as, basal forebrain, cortical regions, hippocampus, amydgala, etc (Ambrogi Lorenzini *et al.*, 1999; Baldi *et al.*, 2000; Myskiw *et al.*, 2010) are involved in processing this kind of memories.

Several assays are available to evaluate spatial learning and memory in rodents, but the Morris water maze, developed at the beginning of the 1980s (Morris, 1984) is the most extensively used (Brandeis *et al.*, 1989; Sharma *et al.*, 2010). In this test the subject is typically placed in a circular pool of opaque water containing either a visible platform or a submerged, hidden platform. The objective is to find the platform using proximal (visible platform task), or distal (hidden platform task) extramaze, cues. Spatial learning is primarily dependent on hippocampal function (Terry, 2009).

Starting from the observations by (Berlyne, 1950) that access to novelty (an object or and environment) can elicit approach behaviours in rats, a new behavioural test was developed in the late 1980s: the so called object recognition test (Aggleton, 1985; Ennaceur & Delacour, 1988). This test is a one-trial task, does not involve learning of rules, does not require reinforces and is purely based on the innate preference of the rodent to explore the novel object. Thus, a rodent that remembers the familiar object will spend more time exploring novel object rather than the familiar one (Leger *et al.*, 2013). A very similar paradigm is the social recognition that measures familiarity recognition. The basic principle relies

on the usual propensity of rodents to investigate an unfamiliar conspecific more thoroughly than a familiar one (van der Kooij & Sandi, 2012). The most widely used variant is the habituation/dishabituation paradigm (Thor & Hollaway, 1982), in which the experimental animal is exposed to an unfamiliar subject. In the retention trial, the animal is presented again to the same subject: recognition memory is indicated by a reduction in the investigation time. The other major variant of this social recognition test is the social discrimination paradigm (Engelmann *et al.*, 1995). The social discrimination paradigm shares the initial exposure to an unfamiliar conspecific with the habituation/dishabituation paradigm but differs in the subsequent phase. In this case, both a familiar and a novel conspecific are simultaneously presented.

Recognition memory judgments may be made using different types of information, including object familiarity, objects spatial location, or when the objects were encountered. Several studies argued that the judgment of prior occurrence for individual items, relies within the perirhinal cortex, while recognition memory that involves the recollection of multiple items and their contextual associations or the temporal order in which items are encountered depends upon interactions between the perirhinal cortex, hippocampus and medial prefrontal cortex (reviewed in Warburton & Brown, 2010). Interestingly, (Barbosa *et al.*, 2013) observed an increase in c-Fos expression in these three brain areas in rats able to discriminate the objects when.tested in the object recognition paradigm.

The role of central histaminergic system in memory and cognition

It is well known that manipulations of the central histaminergic system during several learning paradigms modifies animal behaviour; however, contradictory results were found, as both facilitatory and inhibitory effects of histamine on memory have been described. This is not too surprising, as memory is a complex process that consists of related but dissociable events, involving, in the elaboration of disparate learning situations distinct brain regions activated to different degrees and at different times. The specificity of action of histamine depends on the localization of histaminergic receptor subtypes, the brain regions and the nature of the cognitive task involved, and the activation of specific intracellular pathways (Passani *et al.*, 2007).

Initial studies, conducted in the late 1980's, demonstrated that

intracerebroventricular injection of histamine facilitates consolidation in the rat passive avoidance test (De Almeida & Izquierdo, 1986; 1988). On the other hand, other groups reported that histamine impaired the animal performance in the same task (Zarrindast *et al.*, 2002; Eidi *et al.*, 2003; Zarrindast *et al.*, 2008). When histamine was locally injected into the dorsal and ventral regions of the hippocampus improvements in the rats performance in the inhibitory avoidance test (Alvarez & Banzan, 1996; Alvarez *et al.*, 2001; Da Silva *et al.*, 2006; Alvarez & Banzan, 2008) and also in the radial-maze tasks (Huang *et al.*, 2003; Yamamoto *et al.*, 2007) were observeded. In the social memory task histamine i.c.v. infusion improved, while the inhibition of its synthesis impaired, animal performance (Prast *et al.*, 1996).

The participation of histaminergic receptors on memory modulation was pharmacologically investigated. Administration of H1R antagonists produced different effects on aversive memory tasks: when pyrilamine, diphenydramine, promethazine or chlorpheniramine were administered either systemically (p.o. or i.v.) or centrally (i.c.v.) the results obtained were always an impairment of rat performance on the active avoidance test (Kamei *et al.*, 1990; Kamei & Tasaka, 1991). Oppositely, chlorpheniramine-treated, 31-month-old rats exhibited longer step-through latencies than vehicle-treated old controls, indicative of superior learning of the task (Frisch *et al.*, 1997). It must be noted, though, that there is an age difference in the experimental subjects. The expression pattern of the histamine receptor subtypes could change with age in rodents, and this might account for the opposite effect.

Central infusions of the H2R antagonist cimetidine improved (Zarrindast *et al.*, 2002; Eidi *et al.*, 2003), whereas the H2R agonist 4-methylhitamine impaired rodent performance in the passive avoidance task (Onodera *et al.*, 1998; Zarrindast *et al.*, 2002; Eidi *et al.*, 2003). Da Silva *et al.* (2006) showed that histamine infused into the hippocampal CA1 region immediately after training in the inhibitory avoidance task induced a dose-dependent promnesic effect that was mimicked by the histamine N-methyltransferase inhibitor SKF-91844 as well as by the H2R agonist dimaprit and it was completely blocked by the H2R antagonist ranitidine. The same compounds also reversed the consolidation deficit induced by early maternal deprivation (Benetti *et al.*, 2012). Another H2R agonist, amthamine, infused into the dorsal hippocampus (Giovannini *et al.*, 2003) or into the nucleus

basalis magnocellularis (NBM) immediately after conditioning, improved fear memory expression (Benetti *et al.*, 2013). The histaminergic systems seem to be involved not only in consolidation of fear memory but also in its extinction since intra-CA1 infusion of histamine immediately after non-reinforced retrieval facilitated consolidation of fear extinction in a dose-dependent manner. This facilitation was mimicked by the SKF91488 and dimaprit and reversed by ranitidine, suggesting that fear consolidation and extinction could share some pathways in the hippocampus (Bonini *et al.*, 2011). Recently Benetti & Izquierdo (2013) demonstrated that post-training histamine, SKF-91844 and imetit infusions into the BLA enhanced consolidation while infusions with thioperamide impaired memory consolidation in the inhibitory avoidance.

The injection of ciproxifan, clobenpropit or thioperamide into the basolateral amigdala (BLA) decreased the freezing time of trained rats compared with saline-injected controls in the fear conditioning paradigm thus causing an amnesic effect (Passani *et al.*, 2001). Conversely, intra-BLA injection of (R)-α-methylhistamine and immepip augmented the freezing time, which is an indication of procognitive effects (Cangioli *et al.*, 2002). Post-training H3R blockade with thioperamide or activation with immepip in the NBM potentiateed or decreased, respectively, freezing response at retrieval in the fear conditioning paradigm. Thioperamide induced memory enhancement seems to depend on H2R, but not H1R activation, as the H2R antagonist zolantidine blocked the effect of thioperamide, whereas the H1R antagonist pyrilamine was ineffective (Benetti *et al.*, 2013). The effects of manipulations on the histaminergic system are also studied in spatial and recognition or discriminative memory tests. Blockade of H1R with clorpheniramine improved memory in the water-maze task (Hasenohrl *et al.*, 1999). When infused in the CA1 region immediately, 30 and 120 min post-training, the H1R antagonist, pyrilamine or ranitidine, blocked long-term memory retention in the object recognition test (Da Silveira *et al.*, 2013). On the contrary, the H1R agonist 2-(3-(trifuoromethyl)-phenyl)histamine facilitated learning in the same task (Malmberg-Aiello *et al.*, 2003) Pharmacological blockade of H3R with thioperamide, A-304121, A-317920 or ABT-239 in the adult rat significantly improved (Prast *et al.*, 1996; Esbenshade *et al.*, 2003; Fox *et al.*, 2003; Fox *et al.*, 2005), whereas the H3R agonist immepip impaired social memory (Prast *et al.*, 1996). Similar findings were observed on the object recognition test: H3R antagonists such as

thioperamide, clobenpropit, pitolisant, GSK189254, reversed the amnesic effects induced by scopolamine injections (Giovannini *et al.*, 1999; Ligneau *et al.*, 2007b; Giannoni *et al.*, 2010), while the H3R agonists (R)-α-methylhistamine, imetit and immepip impaired rat performance (Blandina *et al.*, 1996; Da Silveira *et al.*, 2013).

The participation of the central histaminergic system in memory and cognition was also studied using genetically engineered animals (knockout models). It was observed that mice lacking H1Rs or H2Rs display impaired object recognition and spatial memory (Dai *et al.*, 2007), whereas H3R-knockout mice perform better than wild-type mice in a spatial learning task but do not differ from control animals in novel object recognition and passive avoidance learning (Rizk *et al.*, 2004). The results from studies of these three mouse models suggest that histamine facilitates learning and memory. However, histidinedecarboxilase (HDC)-knockout mice show impairment or improvement of cognitive functions depending on the task used to measure learning and memory. For example, HDC-KO mice perform better than wild type mice in the water maze (Dere *et al.*, 2003; Acevedo *et al.*, 2006a) and exhibit greater learning and memory in contextual fear conditioning (Liu *et al.*, 2007). Hippocampal long-term potentiation (LTP) is stronger (Liu *et al.*, 2007; Chepkova *et al.*, 2012) and impairment of LTP by ammonia is also greater in HDC-KO mice than in controls (Chepkova *et al.*, 2012). By contrast, HDC-KO mice show impairments in novel location recognition (Acevedo *et al.*, 2006a) and non-reinforced episodic object memory (Dere *et al.*, 2003).

These are some examples of the great volume of studies regarding the contribution of histaminergic system on mnemonic process. Detailed reviews about the pharmacology and potential therapeutics of histaminergic ligands can be found in: Esbenshade *et al.*, 2008; Kohler *et al.*, 2011; Passani & Blandina, 2011 .

Interactions between the histaminergic and cholinergic systems: effects of H3R modulation

Several evidences support the involvement of the cholinergic transmission as an essential neurophysiological component in cognitive functioning: (i) pharmacological experiments conducted in both animals and humans have shown learning and memory deficits after anticholinergic treatments, (ii) cholinergic disfunction and cell loss have been associated with memory alteration in aged

subjects and Alzheimer's disease patients, which has been related to cognitive deficits, (iii) the clinical efficacy of acetylcholinesterase inhibitors (galantamine, donepezil, rivastigmine) for the cognitive deficits associated with mild to moderate Alzheimer's disease (Micheau & Marighetto, 2011).

In rats, as in humans, projections from the NBM provide the majority of cholinergic innervation to the cortex (Mesulam *et al.*, 1983; Heckers *et al.*, 1994). The administration of H3R antagonists/inverse agonists, such as clobenpropit or thioperamide into the NBM, increased the output of ACh from the cortex of rats implanted with two microdialysis probes, one in the NBM to locally deliver the different drugs, and the other in the cortex to measure the output of ACh. Pretreatment with the H1R antagonist triprolidine into the NBM fully blocks the effect of thioperamide on cortical ACh release (Cecchi *et al.*, 2001). This finding is consistent with a report that histamine depolarizes the membrane and increases the tonic firing of guinea-pig NBM cholinergic neurons through H1R activation (Khateb *et al.*, 1995). This evidence supports the hypothesis that activation mediated by H1R of the dense histaminergic innervation into the NBM facilitates the cholinergic tone in the cortex (Blandina *et al.*, 2004).

Both *in vitro* (Clapham & Kilpatrick, 1992; Arrang *et al.*, 1995), and *in vivo* (Blandina *et al.*, 1996) experiments demonstrated that histamine applied in the cortex decreased the local cholinergic tone, this effect was mimicked by H3R agonists (imetit, immepip and (R)-α-methylhistamine) and prevented by clobenpropit, an H3R antagonist, demonstrating the role of the H3R on this effect. Microdialysis experiments demonstrated that immepip increased GABA release from the cortex of freely moving rats, and bicuculline, a $GABA_A$ receptor antagonist, reversed the inhibition of ACh release induced by immepip (Giorgetti *et al.*, 1997). Thus, it is conceivable that H3 heteroreceptors expressed on cortical GABAergic interneurons facilitate GABA release, which in turn, inhibits ACh release (Blandina *et al.*, 2004). Hence, the histaminergic modulation of cortical cholinergic tone appears to be complex, consisting of two components, one inhibitory, related to local actions at the terminals, the other excitatory, resulting from interactions with cholinergic cell bodies in the NBM.

Consistent with intracerebral applications, systemic administration of H3R antagonists/inverse agonists pitolisant, ABT-239, ABT-288, GSK189254, JNJ10181457 increased cortical ACh release in freely moving rats.

Local perfusion with H3R antagonists/inverse agonists (ciproxifan, clobenpropit and thioperamide) or H3R agonists (immepip and (R)-α-methylhistamine) decreased (Passani *et al.*, 2001) or increased (Cangioli *et al.*, 2002) ACh release from the basolateral amygdala (BLA), respectively. Intra-BLA administration of cimetidine alone increased ACh spontaneous release and fully antagonized the reduction of ACh release induced by the perfusion with H3R antagonists (Passani *et al.*, 2001). In the BLA, H3R is strictly associated with the presence of histaminergic fibers (Anichtchik *et al.*, 2000a), and local perfusion with H3R antagonists/inverse agonists increases endogenous histamine release (Cenni et al. 2003); therefore, the inhibition of ACh release elicited by H3R-antagonists/inverse agonists could be most simply explained by a blockade of inhibitory H3 autoreceptors. H3R antagonists/inverse agonists by blocking the H3 autoreceptors augmented histamine release in the BLA, which, in turn, moderates cholinergic neurotransmission, presumably by activation of postsynaptic H2R, as pretreatment with cimetidine fully antagonized cholinergic inhibition caused by H3R antagonists/inverse agonists. The opposite may occur when H3R are activated (Passani *et al.*, 2001; Blandina *et al.*, 2004).

ABT-239 and GSK189254 systemically injected augmented the hippocampal release of ACh (Fox *et al.*, 2005; Medhurst *et al.*, 2007), but local administration of histamine in the hippocampus did not altered the release of ACh (Bacciottini *et al.*, 2002). Probably, H3R antagonists facilitate the release of ACh in the hippocampus interacting with H3 autoreceptors in the septum where cholinergic cell bodies projecting to the hippocampus are localized (Mesulam *et al.*, 1983; Nicoll, 1985; Gaykema *et al.*, 1990). Using the double probe microdialysis technique it was demonstrated that perfusion with thioperamide and ciproxifan into the medial septum-diagonal band complex (MSA-DB) increased ACh release from the hippocampus, whereas (R)-α-methylhistamine produced the opposite effect. Local perfusion of the MSA-DB with cimetidine moderated the spontaneous hippocampal ACh release and antagonized the facilitation induced by H3R antagonists (Bacciottini *et al.*, 2002). These results indicate a role of endogenous histamine in modulating cholinergic tone in the hippocampus trough interactions with the H2R.

The histaminergic system modulates the activity of cholinergic neurons also in the ventral striatum. Local perfusion with histamine increased the release of

ACh while inhibition of histamine synthesis by perfusion with α-fluoromethylhistidine gradually decreased ACh release. The effect of histamine was inhibited when the striatum was simultaneously infused with the D2/D3 agonist quinpirole and the D1 antagonist SKF 83566 and also by perfusion with the $GABA_A$ receptor antagonist bicuculline (Prast *et al.*, 1999). Local infusion with the H3R antagonist thioperamide enhanced ACh release from the ventral striatum. This effect was abolished after inhibition of histamine synthesis by αFMH and also by simultaneous perfusion with quinpirole and SKF 83566. Imetit and immepip, two H3R agonists also increased ACh release when perfused into the striatum. Their effects of were αFMH resistant, but abolished by simultaneous perfusion with quinpirole and SKF 83566 (Prast *et al.*, 1994; Prast *et al.*, 1999). These findings show that, in the striatum, the activity of cholinergic neurons is modulated by neighboring GABAergic neurons and histaminergic nerve terminals and axons. The enhanced release of acetylcholine by the H3R agonists imetit and immepip seems to be due to stimulation of H3 heteroreceptors, while the increase of ACh release by the H3R antagonist thioperamide could be explained via blockade of H3 autoreceptors (Prast *et al.*, 1999).

AIMS

The H3Rs are auto- and heteroreceptors. *In vitro* experiments showed high degree of constitutive activity and blockade of the H3R was shown to improve rodents performance in several learning tasks. This was accompanied by increased neurotransmitters release, specially histamine and acetylcholine in brain areas involved in cognition like cortex, nucleus basalis magnocellularis and hippocampus, which seems to be responsible for H3R antagonism promnesic effects. We further investigated the interaction between histaminergic and cholinergic systems evaluating the effects of two compounds: ABT-239 (an H3R antagonist/inverse agonist) and donepezil (an acetylcholinesterase inhibitor) in naïve and histamine-depleted (genetically or pharmacologically induced) mice. We used the object recognition test to evaluate the drug-induced procognitive effects because this is a validated task to evaluate animal declarative memories with important cortical and hippocampal cholinergic components (Winters *et al.*, 2008). Moreover, we measured simultaneously the cortical releases of histamine and acetylcholine, by double probe microdialysis in freely moving rats, and the levels of

GSK3β non-phosphorylated and phosphorylated at Ser9 residue in hippocampal and cortical homogenates of normal and histamine-deficient mice by western blot.

The results obtainded suggest that activation of the PI3K/AKT/GSK-3β pathway is a requisite for the procognitive effects elicited by ABT-239 and donepezil in normal mice. Furthermore, the lack of GSK-3β phosphorylation could be responsible for the inefficacy of both drugs on histamine-deficient animals.

METHODS

Animals

Male Sprague Dawley rats (225-275 g; Harlan, Italy) housed in groups of three were used for microdialysis experiments. Male CD1 mice (25-35 g; Harlan, Italy) and histidine decarboxylase knockout (HDC-KO) mice and wild type (WT) littermates (background sv129), grown in the Centro Sabulazione Animali di Laboratorio (CeSAL), Università di Firenze were used for behavioural and biochemical experiments. Animals were housed in humidity and temperature-controlled room (22 - 24 °C) allowed free access to food (4RF21; Mucedola s.r.l., Milan, Italy for mice and Teklad Global 18% protein rodent diet for rats) and water, and kept on a 12-h light/dark cycle (lights start at 7:00 AM). All the experiments were conducted between 9:00 AM and 4:00 PM.

Alternatives to *in vivo* techniques were not available, but all efforts were made to minimize animal suffering and to reduce the number of animals used. Housing, animal maintenance and all experiments were conducted in accordance with the Council Directive of the European Community (86/609/EEC) of the Italian D.L. 116 (1992), NIH guidelines on animal care and approved by veterinarian supervision.

Compounds

ABT-239 and α-fluoromethylhistidine (αFMH) were synthesized at Abbott Laboratories (Abbott Park, USA) and were dissolved in water and physiological saline, respectivelly. Donepezil and neostigmine (Sigma, UK) were dissolved in saline and Ringer's solution, respectivelly. LY294002 (Tocris Bioscience, Bristol, UK) was dissolved in a solution of physiological saline containing 1% of dimethyl sulfoxide (DMSO). All other reagents and solvents were of HPLC grade or the highest grade available (Sigma, UK).

Object Recognition Test (ORT)

Normal and histamine deprived male mice were assessed in a test apparatus comprising an “open-field” arena (60 x 70 cm and 30 cm high) placed in a sound attenuated room. Each mouse was subjected to the procedure separately and care was taken to remove any olfactory/taste cues by cleaning carefully the arena and test objects between trials and mice. Ttwenty four hours after a 10 min habituation period, each mouse was placed into the test arena in the presence of two identical objects (plastic shapes such as cubs, cylinders or pyramids). Each mouse was placed facing the same direction at the same position in the arena, and the time spent actively exploring the objects during a 5 min test period (T1) was recorded. Exploration was defined as sniffing or touching the stimulus object with the nose and/or forepaws. Sitting on or turning around the objects was not considered exploratory behaviour. After 2 or 24 h, during the test session (T2), each mouse was again placed in the test arena for 5 min in the presence of one of the familiar object and a novel object, and the time spent exploring both objects was again recorded. The position of the objects (left/right) was randomized to prevent bias from order or place preference. Mice were placed in its home cage between trials.

Figure II.1 summarizes the different experimental protocols employed in this study. First we evaluated short- and long-term retention memory in normal WT and HDC-KO mice using inter-trial intervals of 2 and 24 hours, respectively (experiment 1). In experiments 2 and 3, we assessed the effects of ABT-239 (3 mg/kg, i.p.) and donepezil (1 mg/kg, i.p.) in acutely or chronically histamine-deficient mice and their respective controls. In experiment 4, we studied the involvement of the PI3K/AKT/GSK3β signalling pathway in the effects of ABT-239 and donepezil in normal mice using the PI3K inhibitor LY294002.

Microdialysis in freely moving rats

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and positioned in a stereotaxic frame. Two guide cannulae (MAB 4.15.IC, Microbiotech, Sweden for HA and CMA12, CMA Midrodialysis, Sweeden) were implanted in the prefrontal cortex according to the following coordinates from bregma (Paxinos & Watson, 1998): AP = + 3.2; L = +0.8; DV = +1.3 at 12° angle for the ACh probe, and AP = + 3.2; L = -0.8; DV = +2.3 at 12° angle for the histamine probe (Figure II.2).

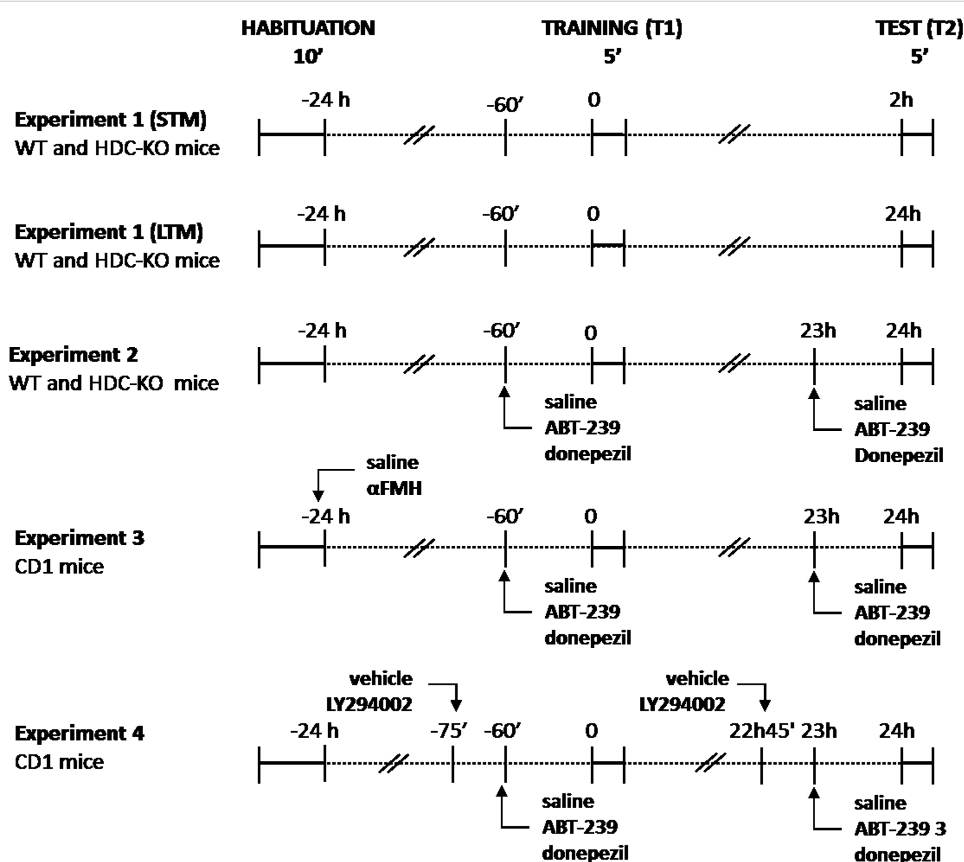

Figure II.1. Schematic representation showing the experimental protocols used to evaluate recognition memory in normal and histamine-deficient animals. The object recognition paradigm consists of three different sessions: habituation (10 min), training (T1, 5 min) and test (T2, 5 min) with intervals of 2 or 24 h between sessions used to evaluate short- and long-term retention, respectively. Treatments: saline, ABT-239 (3 mg/kg) and donepezil (1 mg/kg) were injected i.p. 60 min before training and test sessions. Saline and αFMH (5 µg) were injected i.c.v. immediately after habituation session. Vehicle and LY294002 (40 ng) were injected i.c.v. 75 min before training and test sessions.

The animals were left to recover, housed one per cage, for 48h and then the microdialysis experiments were performed. The stylet was removed from the guide cannulae, and the microdialysis probes were inserted. The probe for ACh (CMA12 Elite, molecular weight cut-off = 20,000 Da, CMA Microdialysis USA) protruded 3 mm from the cannula tip, whereas that for HA (MAB 4.15.2.PES, molecular weight cut-off = 6,000 Da, Microbiotech, Sweden) protruded 2 mm. Probes were perfused with Ringer's solution (in mM: NaCl, 147; $CaCl_2$, 1.2 and KCl, 4.0 at pH 7.0) at a flow rate of 2 µL/min using a microperfusion pump (Carnegie Medicine, Sweden; Mod CMA/100). In the experiments aimed at measuring ACh release, neostigmine bromide (0.1 µM), a cholinesterase inhibitor, was added to the medium perfusing the prefrontal cortex to recover detectable ACh concentrations in the dialysate.

After two hours the sample collection started, at 15 min intervals. Spontaneous release was defined as the average value of the five 15-min fractions collected during 75 min of perfusion with Ringer's solution. The animals then received a single i.p. injection of ABT-239 3 mg/kg. All subsequent fractions were expressed as percentage of this value. In another set of experiments, animals received 5 μg of αFMH into the lateral ventricle (AP = -0.9, L = -1.5, DV = +3.6) during the stereotaxic surgery and 48 h after they were submitted to the same experimental protocol described above. To prevent degradation of HA and ACh,1.5 μL of 5 mM HCl or 5 μL of 0.5 mM HCl were added to each sample, respectively. The dialysates were kept at -80 °C until analysis.

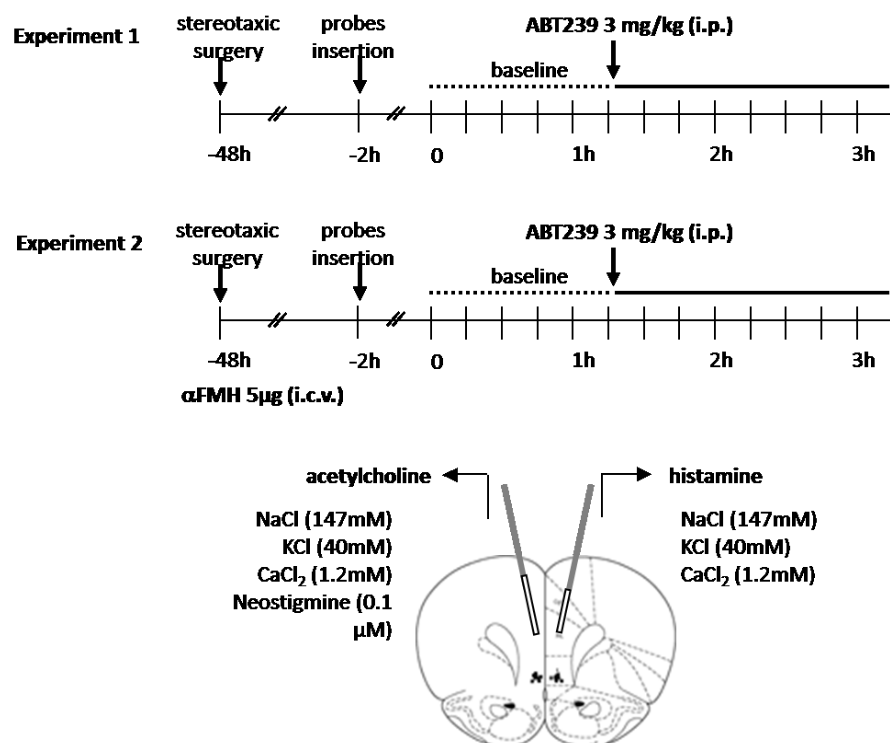

Figure II.2. Schematic diagram showing experimental protocols and the position of the microdialysis probes. Rats were implanted with two contralateral probes in the prefrontal cortex (pFCx) to measure histamine and acetylcholine releases simultaneously. Neostigmine bromide (0.1 uM) was added to the medium to recover detectable ACh concentrations in the dialysate. ABT-239 (3 mg/kg) was systemically injected after collection of 5 baseline samples. In the experiment 2, the animals received 5 μg of alpha-fluoromethylhistidine (αFMH) into the lateral ventricle during the stereotaxic surgery.

Determination of histamine

Histamine contents in the dialysates were determined by HPLC-fluorimetry after post-column derivatization as described in chapter I.

Determination of acetylcholine (ACh)

ACh was assayed in the dialysate by HPLC with electrochemical detection using an ACh/Choline assay chromatographic kit (Bio Analytical System Inc, West Lafayette, Indiana, USA) consisting of an ACh analytical column (BAS MF-6150) and an ACh/Choline Immobilized Enzyme Reactor (IMER, BAS MF-6151). The mobile phase was 50 mM Tris/$NaClO_4$ containing 0.05% ProClin (BAS CF-2150, Bio Analytical Systems Inc, West Lafayette, Indiana, USA), a broad spectrum antimicrobial suited as a preservative for enzymes, pH 8.5, at 1 mL/min flow rate. ACh, separated in the analytical column, was hydrolized in the IMER by acetylcholinesterase to acetate and choline that was oxidized by choline oxidase to produce betaine and hydrogen peroxide. Hydrogen peroxide was electrochemically detected by a platinum-working electrode at +500 mV with an Ag/AgCl reference electrode. The sensitivity limit was 300 fmol, and the signal/noise ratio was higher than 3. To evaluate the amounts of ACh in the samples, a linear regression curve was made with ACh standards, and the peak areas of this compound in the samples were compared with those of the standards by means of an integrator (P.E. Nelson model 1020; The Perkin Elmer Corporation, Norwalk, CT, USA). ACh levels in the dialysate samples were calculated as fmol/15 min, and were not corrected for probe recovery (about 60%).

Histology

The correct placement of microdialysis membranes was verified post-mortem as described in chapter I.

Western Blotting analysis

Male HDC-KO mice and WT littermates received i.p. injections of saline, ABT-239 (3 mg/kg) or Donepezil (1 mg/kg) and 60 minutes later were sacrificed. The brains were dissected on ice, and the cortices and hippocampi isolated immediately. In another set of experiments, 36 CD1 male mice were randomly divided in two groups: half part received an i.c.v injection of 5 µL of saline and the other half part was i.c.v. treated with 5 µL of solution containing αFMH 1µg/µL. Twenty four hours later they were randomly treated with saline (i.p.), ABT-239 3mg/kg (i.p.) or donepezil 1 mg/kg (i.p.) and 60 minutes after they were sacrificed,

the brain dissected on ice and cortices and hippocampi immediately isolated. The cortices and the pooled hippocampi (left and right) were individually homogenized in 200 µL ice-cold lysis buffer containing protease and phosphatase inhibitors (50mM TrisHCl (pH 7.5), 50mM NaCl, 10mM EGTA, 5mM EDTA, 2mM NaPP, 4mM PNFF, 1mM Na_3VO_4, 1.1mM PMSF, 20 µg/µL Leupeptin, 50 µg/µL Aprotinin, 0.1% SDS) and centrifuged at 12000 rpm at 4 °C for 15 minutes. The supernatant was collected and total protein levels were quantified using the Pierce BCA Protein Assay (Thermo Scientific, USA). Homogenates were diluted in a mix of lysis buffer and loading buffer 2x (50mM Tris pH = 6.8, 100mM DTT, 10% Glycerol, 1% Bromophenol Blue, and 2% SDS) and boiled for 10 minutes. Aliquots containing 40 µg total proteins were resolved by electrophoresis on a 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon Transfer Membranes, Millipore, USA). Blots were blocked for 2 h at room temperature in Tris-buffered saline, pH 7.6 containing 0.1% of Tween 20 (TBS-T) containing 5% non-fat dry milk (Bio-Rad Laboratories, USA) and then incubated overnight at 4°C with monoclonal antibodies against phospho-GSK-3β (Ser9) or GSK-3β (both from Cell Signaling Technology, USA) diluted 1:1000 in TBS-T containing 5% bovine serum albumin or 5% non-fat dry milk, respectively. Immunodetection was performed with secondary antibodies (anti-rabbit IgG conjugated to horseradish peroxidase, Cell Signaling technology, USA) diluted 1:5000 in TBS-T containing 1% of non-fat dry milk. Membranes were washed in TBS-T and then reactive bands were detected using enhanced chemiluminescence (Luminata Crescendo, Millipore, USA). Quantitative densitometric analysis was performed using the QuantityOne analysis software (Bio-Rad). For each sample was calculated a ratio of p^{SER9}-GSK-3β/GSK-3β densities and then all the individual rates were expressed as a percentage of the average of ratios obtained from control group.

Statistical Analysis

Statistical analysis was performed using Prism Software (GraphPad Software). The data of the object recognition test are expressed as a percentage of time spent exploring each object during the test session (T2) and statistical significance was determined by the unpaired t-test. Values obtained in microdialysis experiments are expressed as means ± SEM and analyzed with one-

way ANOVA and Bonferroni post-hoc test. For clarity purposes we reported in figures and figure legends only the significant differences vs. the last baseline sample before drug treatment. However, differences were significant versus all baseline samples. Statistical significance for the Western blot analysis was determined using one-way ANOVA followed by Newman Keuls. The level of significance was set at P < 0.05.

RESULTS

Evaluation of short- and and long-term memory in WT and HDC-KO mice

In order to investigate the effect of chronic histamine deprivation on STM and LTM retention we evaluated the performance of WT and HDC-KO male mice on the object recognition test using two inter-trial intervals: 2 and 24 hours. In the test performed 2 hours after the training session, mice from both genotypes recognized the familiar object as they spent significantly higher percentage of time exploring the new object than the familiar one (P<0.001) suggesting that chronic histamine deficiency has no effects on STM retention. However when the test session was performed 24 hours after the training session mice of either genotype were unable to differentiate the novel from the familiar object (Figure II.3).

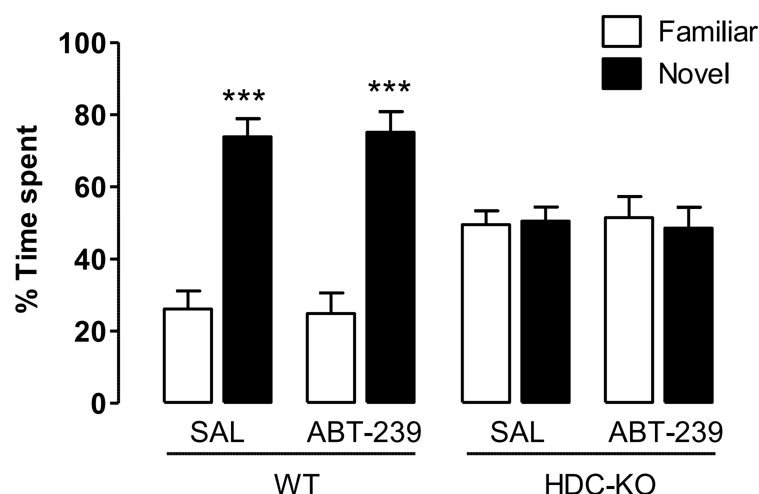

Figure II.3. Effect of chronic histamine deficiency on short and long-term memory retention. Shown are means ± S.E.M. of individual percentage of time spent exploring familiar (white columns) and novel (black columns) objects in the object recognition paradigm with different inter-trial intervals: (A) 2 hours and (B) 24 hours. n = 8-11 animals per experimental group. ***P<0.001 (unpaired *t*-test)

Effects of ABT-239 administration to histamine-deprived mice in the ORT

When tested 24 hs after training, WT mice injected with ABT-239 (3 mg/kg, i.p.) spent significantly more time exploring the new object than the familiar one (P<0.001, unpaired t-test,), whereas no significant differences (P=0.098, unpaired t-test) in the percentage of time spent exploring the familiar object as compared with the novel one were observed in ABT-239-treated HDC-KO mice (Figure II.4A). To avoid biases related to compensatory mechanisms due to chronic depletion of histamine, we assessed the effects of ABT-239 in CD1 mice that received i.c.v. infusions of the HDC suicide inhibitor αFMH or saline. In analogy to the results observed in chronically deprived mice, no differences (P=0.1581, unpaired t-test) were observed in the exploration of objects in acutely histamine-deficient animals that received ABT-239 systemically (Figure II4.B), whereas we observed an increase in the percentage of time spent exploring the new object in ABT-239-treated CD1 control mice (P<0.01, unpaired t-test).

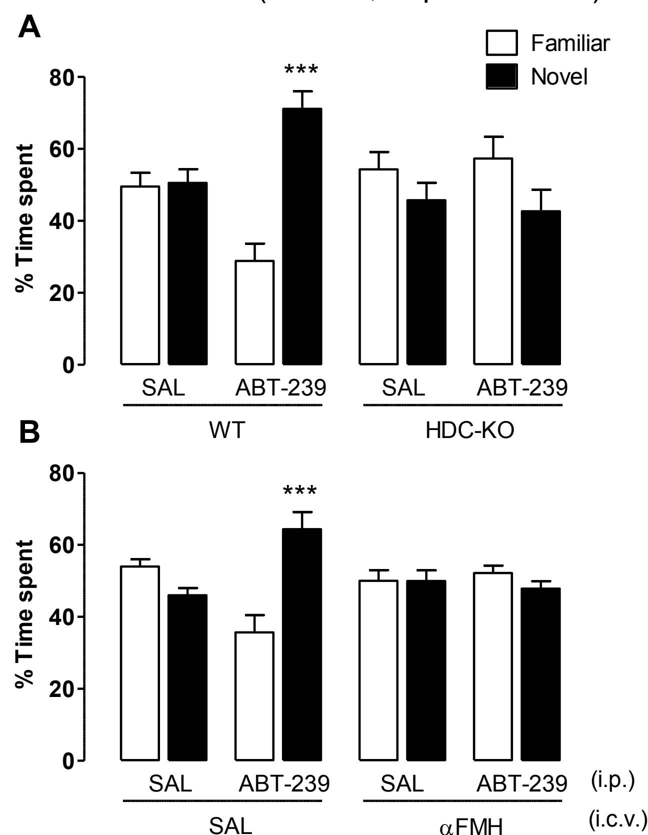

Figure II4. ABT-239 induces procognitive effects in normal but not in histamine-deficient mice in the object recognition test. (A) Effect of systemic administrations of ABT-239 (3 mg/kg, i.p.) or saline to WT and HDC-KO male mice. (B) Effect of systemic administrations of ABT-239 (3 mg/kg, i.p.) in saline or αFMH (5 µg i.c.v.) treated CD1 mice. Systemic treatments were performed 60 min before training (T1) and test (T2), while i.c.v treatments were administrated 24 h before training (T1). Shown are means ± S.E.M. of individual percentage of time spent exploring familiar (white columns) and novel (black columns) objects. n = 10-15 mice per group. **P<0.01, ***P>0.001 (unpaired t-test)

Systemic administration of ABT-239 increased the release of histamine and ACh from the prefrontal cortex of freely moving rats

After collection of five 15-min baseline samples, ABT-239 was administered i.p. at the dose of 3 mg/kg. ABT-239 significantly increased both ACh and histamine release immediately after the administration, up to peak values of 177 ± 21% (ANOVA, $F_{(15,75)} = 4.966$, $P<0.001$), and 132 ± 36% (ANOVA, $F_{(15,75)} = 7.626$, $P<0.001$), respectively. ACh and histamine release remained significantly elevated for about 60 min, and then returned to basal levels (Figure II.5). Mean spontaneous releases were 550 ± 61 fmol/15 min for ACh, and 47 ± 5 fmol/15 min for histamine (n = 6).

In a separate set of experiments we also measured histamine and ACh releases from the rat prefrontal cortex 48h after αFMH (5 µg) i.c.v. injections. Cortical basal histamine and ACh contents of αFMH-treated rats (n = 6) were below the methodological detection limit and no further histamine or ACh releases were elicited when rats were treated with ABT-239 (data not show).

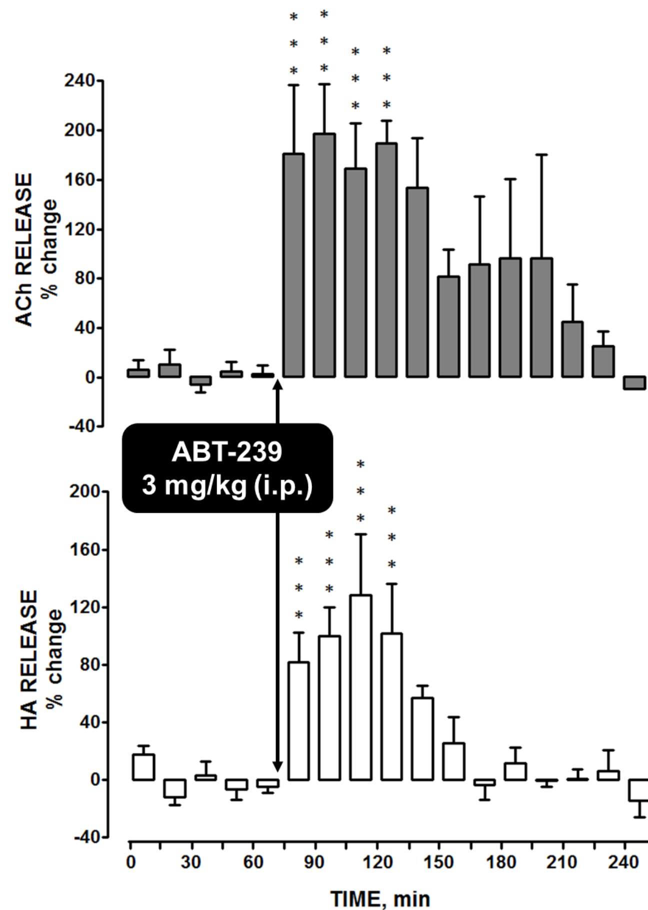

Figure II.5. Time course of ACh and histamine release from the prefrontal cortex of freely moving rats following systemic administration of ABT-239. Neurotransmitters release was measured in fractions collected every 15 min. Neostigmine bromide (0.1 ìM), a cholinesterase inhibitor, was added to the medium perfusing the ACh probe. Control values of spontaneous neurotransmitter release were calculated for each experiment by averaging the mean of five initially collected 15-min samples, and averaged 550 ± 61 fmol/15 min for ACh, and 47 ± 5 fmol/15 min for histamine. Neurotransmitters release was expressed as a percentage of spontaneous release. The arrow indicates the time of drug administration. Represented are means ± SEM of 6 rats. ***P < 0.001 vs. last sample before drug treatment (ANOVA and Bonferroni/Dunn test).

Effects of donepezil administration to histamine-deprived mice in the ORT

As expected, donepezil treatment (1 mg/kg, i.p.) reverted the time-induced amnesic effect as observed by increases in the percentage of time spent exploring the new object with respect to the familiar one in both wild type (P<0.001 unpaired t-test, Figure II.6A) and CD1 mice (P<0.01, unpaired t test Figure II.6B). However, the memory improvement caused by donepezil systemic injection was not observed in acutely or chronically histamine-deficient mice, since no differences between the percentages time spent exploring the familiar and novel objects were observed in HDC-KO (P=0.9137 unpaired t-test, Figure II.6A) nor in αFMH-treated mice (P=0.5440, unpaired t test Figure II.6B).

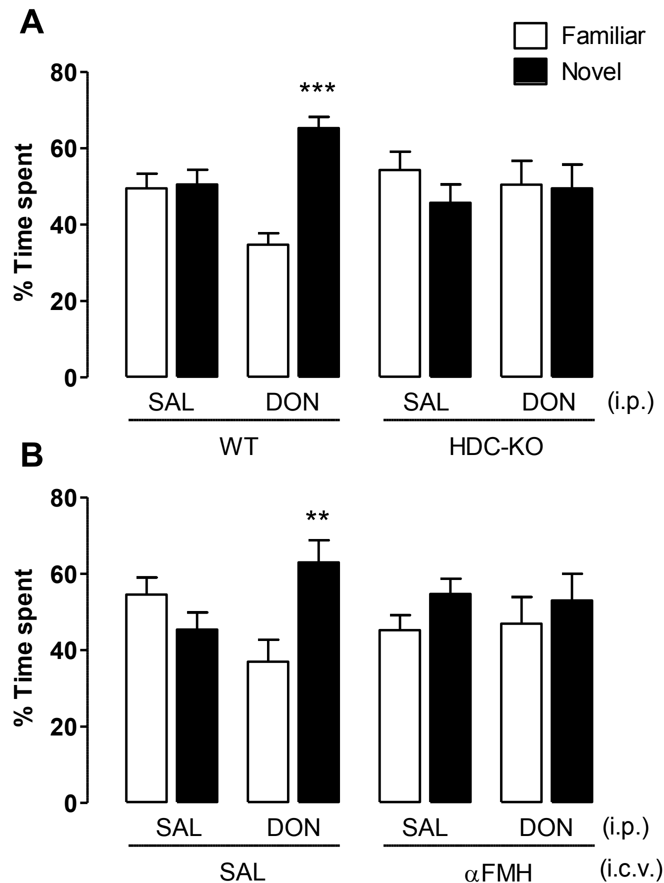

Figure II.6. Donepezil induces procognitive effects in normal but not in histamine-deficient mice in the object recognition test. (A) Effect of systemic administrations of either donepezil (DON, 1 mg/kg, i.p.) or saline to WT and HDC-KO male mice. (B) Effect of systemic administrations of donepezil (DON, 1mg/kg, i.p.) to saline or αFMH (5 µg i.c.v.) treated CD1 mice. Systemic treatments were performed 60 min before training (T1) and test (T2) while i.c.v treatments were administrated 24 h before training (T1). Shown are means ± S.E.M. of individual percentage of time spent exploring familiar (white columns) and novel (black columns) objects. n = 8-15 mice by group. **P<0.01, ***P<0.001, unpaired t-test

Effects of ABT-239 and donepezil on GSK-3β phosphorylation in cortical and hippocampal homogenates from normal and histamine-deprived mice

Using western blot analysis we determined the protein levels of GSK-3β phosphorylated at Ser9 residue in cortical homogenates of normal and histamine-deficient mice treated with either ABT-239 (3 mg/kg, i.p.) or donepezil (1 mg/kg, i.p.) using the same protocol employed for the behavioural test. One way ANOVA revealed an overall difference in cortical homogenates from WT and KDC-KO mice ($F_{(5,35)}$=7.556, P<0.001). Post-hoc comparisons showed a significant increase in p^{Ser9}GSK-3β/GSK-3β ratios after ABT-239 (168.6 ± 28.8%, P<0.001) or donepezil (141.1 ± 9.2%, P<0.05) treatments when compared with saline-treated control

animals. Conversely, no significant changes in GSK-3β phosphorylation were observed on HDC-KO mice that received the same treatments (Figure II.7A). As shown in Figure II.7B, p^{Ser9}GSK-3β/ GSK-3β ratios were also significantly augmented (One way ANOVA $F_{(5,34)}=3.392$, $P<0.05$) in cortical homogenates from CD1 mice treated with ABT-239 (152.7 ± 28.5% $P<0.05$) or donepezil (160.4 ± 22.7%, $P<0.05$). Acute histamine deprivation by means of αFMH central injections prevented this increase (Figure II.7B).

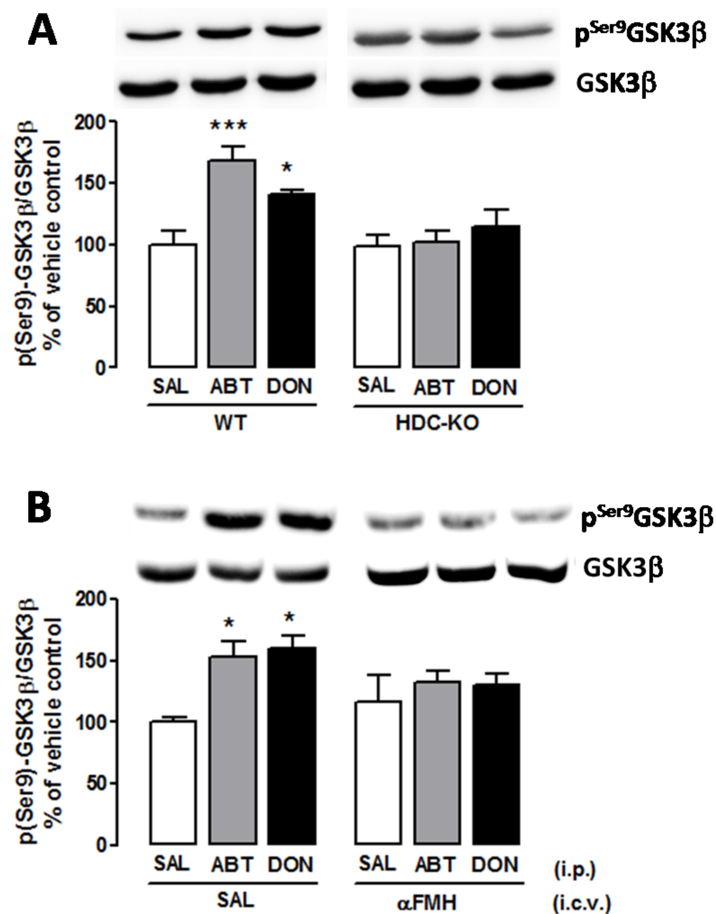

Figure II.7. ABT-239 and donepezil systemic injections increase ^{Ser9}GSK-3β phosphorylation in cortical homogenates from normal but not from histamine-deprived mice. Representative western blots labeled with antibodies against GSK-3β phosphorylated at Ser9 residue and non-phosphorylated GSK-3β of cortical homogenates from chronic (A) and acute (B) histamine-deficient mice treated with saline, ABT-239 (3mg/kg, i.p.) or donepezil (1 mg/kg, i.p.). Shown are means ± S.E.M. of individual values. N = 5-6 mice per experimental group. *P<0.05, **P<0.001 versus saline-treated control mice (basal group), ANOVA with Newman-Keuls multiple comparisons post-hoc test.

The results obtained in hippocampal samples are similar to those observed in cortical homogenates. One way ANOVA ($F_{(5,35)}=4.822$, $P<0.01$) followed by

Newman-Keuls test revealed a statistically significant increase in p^{Ser9}GSK-3β/GSK-3β ratios after ABT-239 (131.7 ± 14.5%, P<0.05) or donepezil (145.1 ± 9.3%, P<0.05) treatment in wild type mice as compared to the saline-treated animals of the same genotype. No differences in p^{Ser9}GSK-3β/GSK-3β ratios were observed in HDC-KO littermates that received the either ABT-239 or donepezil (Figure II.8A). Systemic administration of ABT-239 or donepezil determined also significant increases in p^{Ser9}GSK-3β/GSK-3β ratios (One Way ANOVA $F_{(5,35)}$=5.645, P<0.001) in hippocampal homogenates from CD1 mice: ABT-239 = 152.9 ± 7.6%, P<0.01; donepezil = 144.4 ± 24.5%, P<0.05 Results observed with acute histamine-deprived mice mirrored those observed in chronically-deficient mice (Figure II.8B).

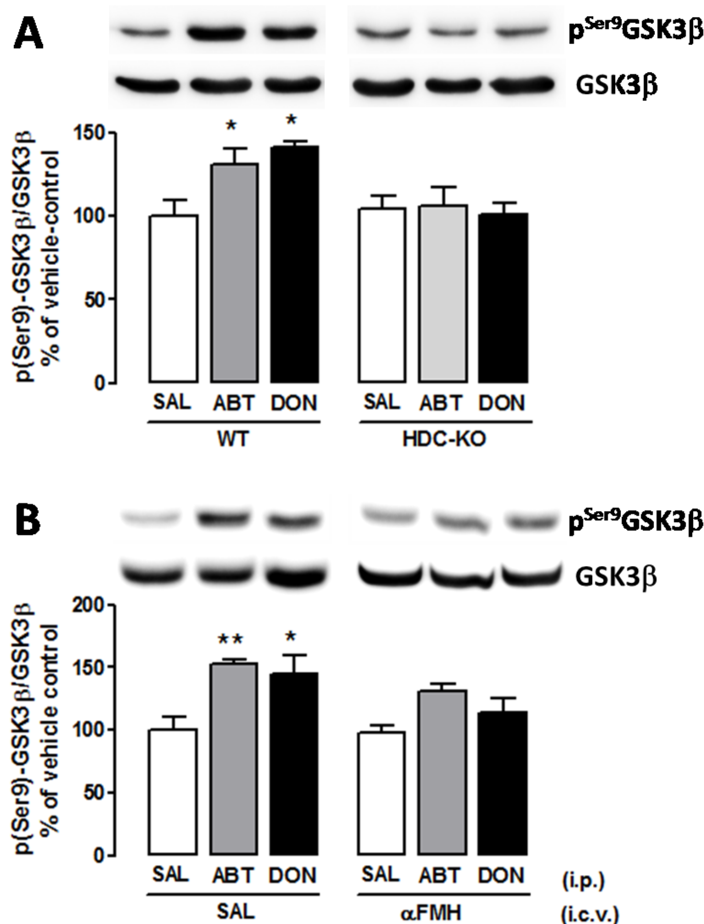

Figure II.8. ABT-239 and donepezil systemic injections increase p^{Ser9}GSK-3β phosphorylation in hippocampal homogenates from normal but not from histamine-deprived mice. Representative western blots labeled with antibodies against GSK-3β phosphorylated at Ser9 residue and GSK-3β non-phosphorylated of hippocampal homogenates from chronic (A) and acute (B) histamine-deficient mice treated with saline, ABT-239 (3mg/kg, i.p.) or donepezil (1 mg/kg, i.p.). Shown are means ± S.E.M. of individual values. n = 5-6 mice by experimental group. *P<0.05, ***P<0.01 versus saline-treated control mice (basal group), ANOVA with Newman-Keuls multiple comparisons post-hoc test.

It is important to note that histamine depletion *per se* did not induced changes in GSK-3β phosphorylation, since no differences were observed between genotypes in saline-treated mice, neither between CD1 mice that received i.c.v. injections of saline or αFMH.

Pre-treatment with LY294002 abolishes ABT-239 and donepezil-induced effects in the ORT.

To test the hypothesis of the involvement of GSK3β phosphorylation in the procognitive effects of ABT-239 and donepezil, we assessed the effects of a central injection of a PI3K inhibitor, LY294002. Vehicle (1% DMSO in physiological saline) or LY294002 (40 ng, equivalent to 25 µM in 5µL) were administered i.c.v. 15 minutes before the systemic treatments in CD1 mice. LY294002 dose was chosen based on previous works (Barros *et al.*, 2001). As expected, systemic injections of ABT-239 (3 mg/kg, i.p.) and donepezil (1 mg/kg, i.p.) increased the percentage of time spent exploring the new object compared to the familiar one in mice that received i.c.v. injections of vehicle ($P<0.05$; unpaired t-test), however, pre-treatment with LY294002 prevented the memory enhancing effects elicited by ABT-239 and donepezil (Figure II.9).

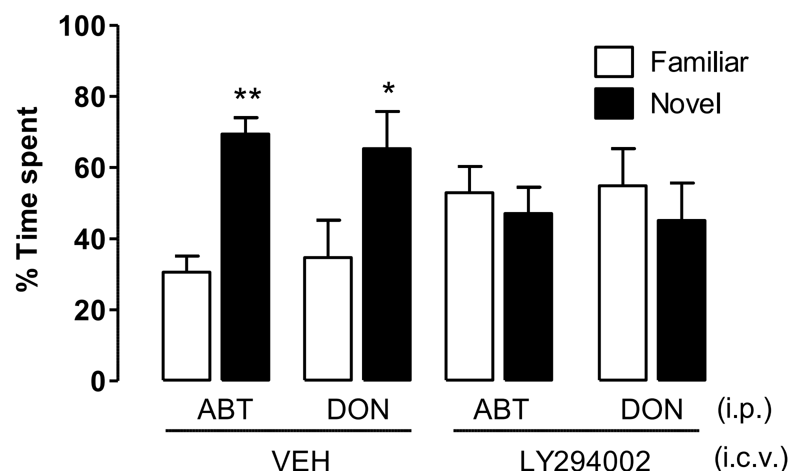

Figure II.9. Inhibition of PI3K/AKT/GSK3β intracellular pathway prevents the ABT-239 and donepezil procognitive effects in the mice object recognition test. LY294002 (40 ng) or vehicle were i.c.v. injected 75 min before training (T1), while systemic treatments with AB-T239 (3 mg/kg, i.p.) or donepezil (1 mg/kg, i.p.) were performed 60 min before T1 and test (T2) sessions. Shown are means ± S.E.M. of individual percentage of time spent exploring familiar (white columns) and novel (black columns) objects. n = 8-9 mice by experimental group. *P<0.05 (unpaired t-test)

Effects of ABT-239 and donepezil treatments on general motor activity of normal and histamine-deprived animals.

General motor activity was measured as the time spent moving in the arena during the 5 minutes of the test session (T2) and monitored during all object recognition protocols. As shown in table II.1, no significant differences were observed among the experimental groups within the experiments.

TABLE II.1. Effects of ABT-239 (3mg/kg, i.p.) or donepezil (1 mg/kg) treatments to normal and acute (αFMH treated) or chronic (HDC-KO) histamine-deprived animals. Motor activity is expressed as mean ± S.D. of the time spent, in seconds (s), moving on the arena during the test session (T2). Treatments: saline, ABT-239 (3 mg/kg) and donepezil (1 mg/kg) were injected i.p. 60 min before training and test sessions. Saline and αFMH (5 µg) were injected i.c.v. immediately after habituation session. Vehicle (DMSO 1%) and LY294002 (40 ng) were injected i.c.v. 75 min before training and test sessions.

	Animals	Treatments		Inter trial	Motor
		i.c.v.	i.p.	interval (h)	activity (s)
Experiment #1	WT	-	-	2	105.4 ± 49.6
	HDC-KO	-	-	2	100.7 ± 51.6
	WT	-	-	24	112.5 ± 14.1
	HDC-KO	-	-	24	108.7 ± 25.0
Experiment #2	WT	-	saline	24	122.5 ± 40.1
	WT	-	ABT-239	24	127.9 ± 69.2
	WT	-	donepezil	24	110.5 ± 16.3
	HDC-KO	-	saline	24	143.5 ± 49.2
	HDC-KO	-	ABT-239	24	132.6 ± 41.1
	HDC-KO	-	donepezil	24	120.1 ± 14.3
Experiment #3	CD1	saline	saline	24	154.6 ± 28.7
	CD1	saline	ABT-239	24	135.9 ± 15.0
	CD1	saline	donepezil	24	158.2 ± 45.2
	CD1	áFMH	saline	24	135.4 ± 27.8
	CD1	áFMH	ABT-239	24	145.5 ± 19.8
	CD1	áFMH	donepezil	24	148.6 ± 28.4
Experiment #4	CD1	vehicle	ABT-239	24	147.2 ± 42.7
	CD1	vehicle	donepezil	24	132.7 ± 41.8
	CD1	LY294002	ABT-239	24	129.3 ± 23.8
	CD1	LY294002	donepezil	24	145.6 ± 37.1

DISCUSSION

The generation of HDC gene knockout mice, lacking functional HDC enzyme and therefore unable to synthesize histamine (Ohtsu *et al.*, 2001) constituted a very interesting tool to investigate the function of endogenous histamine in the brain. HDC-KO mice show a complex behavioral phenotype including decreased attention, waking and novelty induced arousal (Parmentier *et al.*, 2002), reduced general motor activity specially during the dark period (Kubota *et al.*, 2002), normal habituation to a novel environment (Dere *et al.*, 2004) and increased levels of anxiety (Dere *et al.*, 2004; Acevedo *et al.*, 2006a; Acevedo *et al.*, 2006b). In our work we did not find differences between HDC-KO mice and WT littermates in the object recognition test: when the test was performed 2 hours after the training section we observed that both WT and HDC-KO mice spent more time exploring the new object with respect to the familiar one. This memory was short lived, as no differences were observed in the time spent exploring the novel and familiar objects when mice were tested 24 hours after training. Our results are in agreement with Acevedo *et al.* (2006b) as they described a deficit of HDC-KO mice in locating the novel object, but not in novel object recognition: when the position of a familiar object in the arena was changed WT mice spent more time in the new as compared to the familiar zones, whereas HDC-KO mice spent similar times in all zones. In a second test, when one of the familiar objects was changed for a new one in the same location both genotypes spent more time exploring the new object. On other hand Dere *et al.*, 2003 described impairments on object recognition memory in HDC-KO mice. These authors used a very complex protocol with several training and tests sessions in which the mice were presented with 4 objects and were demanded to discriminate the objects based on the sequence of presentation. The number of contacts with the objects was considered to measure memory retention. WT mice showed higher numbers of contacts with the most recent objects respect to the familiar ones and this was not observed for HDC-KO mice. However, the authors observed that the sum of the contacts made by HDC-KO mice were lower as compared with the WT animals and this could account for the results observed.

The procognitive effect of ABT-239 systemic injection was observed in WT but not in HDC-KO littermates. This behavioural response could be explained by a reduction of H3R mRNA expression in the hippocampus of HDC-KO mice as

reported by (Chepkova *et al.*, 2012). To evaluate this possibility we assessed the effect of ABT-239 in mice acutely deprived of brain histamine with i.c.v. injections of αFMH a highly specific irreversible inhibitor of histidine decarboxylase whose administration causes a rapid depletion of histamine levels in the mouse brain (Maeyama *et al.*, 1982). In a similar way to that observed in HDC-KO mice, ABT-239-treated mice were unable to discriminate between novel and familiar objects.

According to previous works (Fox *et al.*, 2005), here we demonstrate, using double-probe microdialysis, that a single i.p. injection of ABT-239 induces a rapid and transient increase of both histamine and ACh from the prefrontal cortex of freely moving rats. The increased ACh release may follow the blockade of cortical H3 heteroreceptors or alternatively to increased histamine release in the NBM, that in turn activates H1Rs on cholinergic neurons innervating the cortex. This would result in increased cortical release of ACh as described by Cecchi *et al* (2001). Test conditions like our in which cholinesterase inhibitors are present in the perfusion medium may be criticized because cholinesterase inhibition might affect ACh spontaneous release. Indeed, neostigmine changed the effect of dopaminergic agents on striatal ACh release (DeBoer & Abercrombie, 1996). However spontaneous cortical ACh release is significantly lower compared with striatal basal release, hence accurate measurements required the presence of cholinesterase inhibitors in the perfusion fluid. Infact, microdialysis studies measuring histaminergic (Blandina *et al.*, 1996; Cecchi *et al.*, 2001; Fox *et al.*, 2005), serotonergic (Consolo *et al.*, 1996), glutamatergic (Giovannini *et al.*, 1994) and GABAergic (Giorgetti *et al.*, 2000) modulation of cortical ACh release invariably employed cholinesterase inhibitors. Relevant in this regard is the observation that concentrations up to 0.5 µM neostigmine did not altered qualitatively the cortical ACh release patterns in response to tactile stimulation (Himmelheber *et al.*, 1998). Following oral dosing, GSK189254, another H3R antagonist, induced a significant increase in ACh release from the anterior cingulate cortex (Medhurst *et al.*, 2007). These experiments were performed without cholinesterase inhibitors in the perfusing medium, but microdialysis samples were collected every 30 min instead of 5 e 15 min as in the case of the other studies. Therefore, it is unlikely that the presence of 0.1 µM neostigmine in the perfusion medium as in our study affects significantly the effect of ABT-239 on cortical ACh release. In the second microdialysis experiment the pre-treatment

with α-FMH completely suppressed both spontaneous and ABT-239-evoked histamine releases, as expected. Surprisingly, the ACh basal and evoked levels were under our methodological detection limit, suggesting the involvement of the histaminergic system in the regulation of basal cholinergic tone in the cortex.

Several findings strongly suggest that H3R-elicited cognitive improvements or deficits are associated with changes in ACh release depending on whether H3Rs are blocked or activated. To have more insight about the interaction between histaminergic and cholinergic systems in memory and cognition we investigated the effects of donepezil, an acetylcholinesterase inhibitor used in clinics to treat mild cognitive deficits observed in AD patients (Lyle *et al.*, 2008). Interestingly and in analogy with ABT-239, donepezil-induced procognitive effect was absent in acute and chronic histamine-deficient mice. Little is known about the cholinergic system of HDC-KO mice. Dere *et al.* (2004) found higher ACh concentration in the frontal cortex and reduced content in the neo striatum and no significant alterations in the ventral striatum, basolateral amygdala and hippocampus as measured in homogenates from these regions. These results suggest that the interaction between cholinergic and histaminergic systems is more complex than previously believed and further experiments are required to fully understand its mechanism and the physiological implications. A crucial experiment will be measuring histamine and ACh release in HDC-KO and WT mice after donepezil systemic treatments. Unfortunately, technical limitations make this experiment unfeasible in the mice.

In the central nervous system PI3K/AKT/GSK-3β axis plays a prominent role in several brain functions, for instance, disruption of this signaling pathway by means of local injections of the PI3K inhibitor LY294002 results in impairments of acquisition, consolidation and retrieval of memory in diverse tasks (Barros *et al.*, 2001; Chen *et al.*, 2005; Sui *et al.*, 2008; Slouzkey *et al.*, 2013). It is demonstrated that object training alone rapidly increased hippocampal GSK3β phosphorylation (Fortress *et al.*, 2013) and transgenic mice with high expression of the phosphorylation defective constitutively active GSK-3β[S9A] isoform showed impaired recognition memory (Dewachter *et al.*, 2009). Moreover, changes in GSK-3β phosphorylation state, expression levels and increased activity were found in brains of Alzheimer's disease patients (Hye *et al.*, 2005; Leroy *et al.*, 2007; Hooper *et al.*, 2008). Recent data showed that GSK-3β hyperactivity leads

to tau hyper-phosphorylation and subsequent intracellular neurofibrillary tangles formation, increased β-amyloid production and memory impairment (Giacobini & Becker, 2007; Hooper *et al.*, 2008; Jaworski *et al.*, 2011). Here we demonstrate that the systemic injections with ABT-239 and donepezil increased the p^{Ser9}GSK-3β/GSK-3β ratios in cortical and hippocampal homogenates from normal mice in a time-course comparable with the behavioural test. However, this effect was not observed in histamine-deficient mice. Moreover, central injection of LY294002, prevented the effects of both drugs in the object recognition test, demonstrating the importance of PI3/AKT/GSK-3β intracellular pathway for the procognitive effects induced by these compounds.

Previous works demonstrated that ABT-239 systemic injection increased phosphorylation of the Ser9 residue of GSK-3β in the cingulate cortex following acute administration to normal CD1 mice. Moreover, 2 week subcutaneous infusion of ABT-239 reverted the reduced p^{Ser9}-GSK-3β hippocampal expression (hence hyperactivation) in APP-overexpressing Tg2576 mice, a transgenic model of Alzheimer's disease (Bitner *et al.*, 2011). However, studies in cultured embryonic neurons demonstrated that H3R activation results in AKT and GSK-3β phosphorylation (Bongers *et al.*, 2007b; Mariottini *et al.*, 2009). In this regard, H3R antagonist neurotransmitter release and subsequent postsynaptic receptor stimulation, not present in an *in vitro* system, may be responsible for the distinct signalling phenotype observed *in vitro* and *in vivo.* In the same work Bitner *et al.*, (2011) reported no significant increase of p^{Ser9}-GSK-3β in the cingulate cortex of CD1 mice after donepezil systemic treatment However, other acetylcholinesterase inhibitors like physostigmine (De Sarno *et al.*, 2006) and phenserine (Ponce-Lopez *et al.*, 2011) systemic treatments are shown to increase cortical and hippocampal GSK-3β phosphorylation in analogy to observed with donepezil in our work. Moreover it was demonstrated that donepezil treatment increased the phosphorylation of AKT (Autio *et al.*, 2011), one of the upstreams modulators GSK-3β, further supporting our results.

In vitro studies showed that donepezil have protective effects against β-amyloid-induced neuronal toxicity inhibiting GSK-3β activity through the PI3K/AKT pathway because LY294002 blocked this effect. Activation of nAChr seems to be involved since mecamylamine partially blocked donepezil effects (Noh *et al.*, 2009; Noh *et al.*, 2013). On other hand, ABT-239 also stimulates p^{Ser9}-GSK-3β

expression in animals lacking the major nicotine acetylcholine receptor in the brain, i.e., α7 nAChR-KO mice suggesting that ABT-239-induced GSK-3β phosphorylation does not require α7 nAChR activation (Bitner *et al.*, 2011). Thus participation of other receptors such as muscarinic receptors (MR) could contribute to this effect. In this regard, it was demonstrated that functional selective muscarinic receptor agonists like pilocarpine, AF267B, Lu25-109 and SKB20226 increased GSK-3β phosphorylation both *in vitro* (Forlenza *et al.*, 2000; Farias *et al.*, 2004) and *in vivo* (De Sarno *et al.*, 2006). Further work is needed to clarify the involvement of these receptors on ABT-239 and donepezil-evoked GSK-3β phosphorylation and cognitive effects.

Interestingly, loss of basal forebrain cholinergic neurons via 192-IgG saporin-induced degeneration increased GSK-3β activity, via decreased Ser9 phosphorylation (Hawkes *et al.*, 2005). Altogether these data point for the importance of the cholinergic system for the GSK-3β phosphorylation and its involvement in neuroprotection and cognition. So, in theory, impaired cholinergic transmission observed in histamine-deprived animals could be related to the lack of both drug-induced GSK-3β phosphorylation and cognitive performance.

CONCLUSIONS

Taken together the results presented in this chapter points to the integrity of the central histaminergic system as a requirement for the biochemical and behavioural effects elicited by two procognitive compounds: ABT-239 and donepezil. Moreover, we demonstrated the involvement of the PI3K/AKT/GSK-3β intracellular pathway in the memory improvement induced by these drugs on the object recognition test and we hypothesize that cortical and hippocampal GSK-3β phosphorylation deficiency is responsible for the inefficacy of ABT-239 and donepezil in acute and chronic histamine-deficient mice.

CHAPTER III

The satiety factor Oleoylethanolamide recruits the brain histaminergic system to inhibit food intake

Body weight is tightly regulated by complex homeostatic mechanisms controlling the balance between food intake and energy expenditure, even subtle mismatches (less than 0.5%) in this balance are sufficient to cause weight gain (Rosenbaum *et al.*, 1997). Thus, obesity can be defined as a state in which energy intake chronically exceeds energy expenditure, and is widely recognised as a largest and fastest growing public health problem in developed and developing world. In fact the World Health Organization created the neologism "globesity" to define a growing global epidemic of overweight and obesity.

Worldwide obesity has nearly doubled since 1980. It has been estimated that in 2008, more than 1.4 billion adults were overweight and over 200 million men and nearly 300 million women were obese (WHO, 2008). Data from the Istituto Nazionale di Statistica revealed that in Italy the prevalence of obesity among adults was 10.3% in 2010, one of the lowest within European countries (ISTAT, 2012).

Obesity etiology is not completely understood, but for sure environmental and lifestyle factors such as increased access to high caloric and unbalanced food, reduction of physical activity, and also genetic predispositions contribute in favor to the deposition of calories as fat (Rosenbaum *et al.*, 1997; Barsh & Schwartz, 2002; Suzuki *et al.*, 2012).

There are innumerable complications in obese patients: type 2 diabetes, metabolic syndrome, hypertension, dyslipidaemia, myocardial infarction, stroke, certain cancers, sleep apnea and osteoarthritis, leading to substantial increases in morbidity, premature mortality and impaired quality of life and others (Hagan & Niswender, 2012). Obesity is also associated with large healthcare costs, in 2006 it was estimated that the Unites States illness-related economic costs exceed US$ 100 billion per year (Daniels, 2006). Although prevention through education and changes in the life style associated with psychological therapies are the first line choices, they are not effective in all patients. A complementary strategy is the pharmacological therapy, but unfortunately, available anti-obesity drugs are scarce and some were hastily withdrawn from market owing to unacceptable side-effects burden (Rodgers *et al.*, 2012). This way a better understanding of all the factors controlling feeding behaviour could lead to the identification of new targets for development of really innovative drugs with better safety and efficacy profiles.

The role of the gut/brain axis in modulating food intake

The gastrointestinal tract and adipose tissue release more than 20 different regulatory peptide hormones that influence a number of physiological processes. The release of gut hormones is stimulated by distension of the stomach and interactions between nutrients and the luminal wall of the intestine (Konturek *et al.*, 2004). In addition to local paracrine actions and peripheral endocrine effects, these hormones play a pivotal role relaying information on nutritional status to important appetite controlling centres within the CNS, such as hypothalamus and the brainstem, which integrates this peripheral information with higher brain centre signals (e.g. reward and mood) and contribute to regulate feelings of hunger and satiety (Sam *et al.*, 2012; Suzuki *et al.*, 2012). There are several central and peripheral stimuli building a very complex network orchestrating feeding behaviour, in the follow sections are reviewed the most important factors.

Neural Pathways related to Appetite Control

Feeding and energy expenditure are controlled by complex neuronal networks distributed through the forebrain and brainstem. Reward-related brain regions have been implicated in the non-homeostatic control of feeding whereas homeostatic feeding behaviour is integrated within the hypothalamus (Benarroch, 2010b). Hetherington & Ranson (1940) and Anand & Brobeck (1951) were the first to propose the existence of hypothalamic centres with opposite functions: a lateral hypothalamic area acting as a "feeding or appetite centre" and ventromedial hypothalamus working as "satiety centre". Subsequent studies demonstrated also a role of other hypothalamic nuclei such as arcuate nucleus (ARC), paraventricular nucleus (PVN), dorsomedial and ventromedial hypothalamus (DMH and VMH, respectively) and lateral hypothalamic area (LH) in energy homeostasis. However, rather than specific hypothalamic nuclei controlling energy homeostasis, it is now thought to be regulated by neuronal circuits (Stanley *et al.*, 2005; Suzuki *et al.*, 2012).

The neuropeptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) are co-expressed in the ARC neurons (Koylu *et al.*, 1997). Cleavage of POMC produces alpha-melanocyte-stimulating hormone (alpha-MSH) which binds to melanocortin-3 (MC3R) and melanocortin-4 (MC3R) receptors to suppress food intake. From the five melanocortin receptors

identified, MC3R and MC4R are likely to play a role in energy homeostasis. They are widely expressed in the hypothalamus and are found in the ARC, VMH, and PVN (Mountjoy *et al.*, 1994; Harrold *et al.*, 1999). Absence of MC4R results in hyperphagia and obesity in rodents (Fan *et al.*, 1997; Huszar *et al.*, 1997), and abnormalities of this receptor have been implicated in 1–6% of severe early-onset human obesity (Farooqi *et al.*, 2000; Lubrano-Berthelier *et al.*, 2003a; Lubrano-Berthelier *et al.*, 2003b). Although MC4R involvement in regulation of feeding is well established, the role of MC3R remains unclear. MC3R mutations have been reported in morbidly obese human subjects (Tao, 2010) and with high-fat diet MC3R-deficient mice develop obesity and have a further increase in adipose tissue compared with wild-type littermates (Chen *et al.*, 2000), however, relatively selective MC3R agonists fail to suppress feeding (Abbott *et al.*, 2000).

CART is the third most abundant transcript identified within the hypothalamus, expressed in the ARC (co-localized with POMC), LH and PVN (Couceyro *et al.*, 1997). CART administration into the lateral ventricles suppresses feeding, whereas injection of CART antiserum increases food intake (Kristensen *et al.*, 1998). However, CART injected into discrete hypothalamic nuclei of fasted rats increased food intake (Abbott *et al.*, 2001), suggesting opposite effects depending on the site of administration, thus, the physiological role of CART in energy homeostasis remains unclear (Suzuki *et al.*, 2012).

Two endogenous antagonists of MCRs have been described: agouti and agouti-related protein (AgRP). Agouti expression is normally restricted to hair follicles where its antagonist effect on the peripheral MC1R results in a yellow pigment (Slominski *et al.*, 2004). Unlike agouti, AgRP is expressed in the CNS, primarily in the medial part of the ARC nucleus (Shutter *et al.*, 1997) and is a potent selective antagonist at MC3R and MC4R (Ollmann *et al.*, 1997). AgRP mRNA expression is increased by fasting (Swart *et al.*, 2002) and central administration of AgRP is able to block alpha-MSH-induced anorexia and increase nocturnal food intake (Rossi *et al.*, 1998). Moreover, AgRP may increase body weight via decreased energy expenditure since repeated central administration of AgRP reduces oxygen consumption, and decreases the ability of brown adipose tissue to expend energy (Small *et al.*, 2001; Small *et al.*, 2003). Transgenic mice overexpressing AgRP are obese (De Backer *et al.*, 2011) whereas reduction of hypothalamic AgRP by RNA interference reduces body weight (Makimura *et al.*,

2002). The role of AgRP in human obesity is less clear, although a polymorphism in the AgRP gene in humans has been reported to be associated with reduced body weight and fatty mass (Marks *et al.*, 2004).

In the ARC nucleus AgRP is co-expressed with another orexigenic peptide, Neuropeptide Y (NPY). NPY/AgRP neurons extensively project to the adjacent hypothalamic nuclei such as PVN, DMH and LH (Broberger *et al.*, 1998b). NPY is one of the most abundant neuropeptide in the brain (Allen *et al.*, 1983). Hypothalamic levels of NPY reflect the body's nutritional status with hypothalamic NPY mRNA and NPY release increasing with fasting and decreasing after refeeding (Sanacora *et al.*, 1990; Swart *et al.*, 2002). Single NPY Injections into the third ventricle stimulated food intake in rats (Clark *et al.*, 1984). Repeated bilateral PVN injections for 10 days increased two-fold daily food intake and six-fold the rate of body weight gain (Stanley *et al.*, 1986). The orexigenic effect of PNY appears to be mediated by stimulation of hipothalamic Y1 and Y5 receptors, whereas Y2 and Y4 receptors are presynaptically localized and have an autoinhibitory effect on NPY neurons (King *et al.*, 1999; King *et al.*, 2000).

The LH also receives projections form ARC and contains two orexigenic neuropeptides, melanin-concentrating hormone (MCH) and orexin (Ox-A and Ox-B) also referred as hypocretin (1 and 2) (Broberger *et al.*, 1998a). Initial studies showed that central Ox injections induce feeding in rats and mice (Sakurai *et al.*, 1998; Edwards *et al.*, 1999). Conversely, central administration of SB334867, a selective Ox receptor-1 antagonist, greatly reduced food intake in rats (Haynes *et al.*, 2000). Both central and peripheral stimuli have been shown to modulate Oxs actions on feeding behavior (reviewed in Sellayah & Sikder, 2013). Increased glucose concentrations inhibit Ox neuronal firing, reducing feeding (Yamanaka *et al.*, 2003), whereas decreased glucose concentrations increased firing rate of orexin-producing neurons, which promote feeding (Burdakov *et al.*, 2005). It was demonstrated that centrally injections of Ox-A increased NPY expression in the ARC (Lopez *et al.*, 2002). Recently, Kay *et al.*, 2014 demonstrated that central injections of Ox-A increased palatable food intake and operant response for food, both effects blocked by the Ox1R antagonist, which also disrupts the conditioned place preference induced by palatable food, suggesting that endogenous stimulation of OX1Rs contributes at least in part some aspects of food reward.

Oxytocin (OXY) is a neuropeptide produced in the magnocellular cells of the

SON and the PVN of the hypothalamus and is released into the systemic circulation from axon terminals in the neurohypophysis (Sofroniew, 1983). OXY is well known for its ability to stimulate milk ejection and uterine contraction but evidence is accumulating in favor of its involvement also in several physiological and pathological functions such as antinociception, anxiety, social recognition, stress responses and feeding (reviewed in Hashimoto *et al.*, 2012). OXY administration into the lateral ventricles inhibited feeding in hungry rats, which was prevented by co-administration of L-368,899 an OXY-R antagonist (Olson *et al.*, 1991; Verbalis *et al.*, 1993). Accordingly, OXY-KO mice consumed larger quantities carbohydrates than wild-type mice (Sclafani *et al.*, 2007). It was demonstrated that several central and peripheral satiety factors such as cholecystokinin (CCK), prolactin-releasing peptide (PrRP), Nesfatin-1 and CART can modulate OXY neuronal activation and release, thus contributing to its hypophagic effects (reviewed in Hashimoto *et al.*, 2012). Maejima *et al.*, 2011 showed that peripheral OXY treatment reduced food intake and visceral fat mass. Peripheral OXY treatment provides a new therapeutic avenue for treating obesity and hyperphagia. In fact, intranasal oxytocin reduces reward-driven food intake in healthy humans (Ott *et al.*, 2013). Although it is still unclear the pathophysiological role of OXY in eating disorders, it was observed in a post mortem study, that in patients with the Prader–Willi syndrome, characterized by extreme hyperphagia leading to morbid obesity, the number of OXY-containing neurons was decreased in the PVN (Swaab *et al.*, 1995). Recent studies showed that overnight OXY secretion was decreased in women with anorexia nervosa as compared with healthy women but postprandial oxytocin secretion was higher in women with active anorexia nervosa and lower in weight-recovered women with anorexia nervosa compared with healthy women (Lawson *et al.*, 2012; Lawson *et al.*, 2013)(Lawson et al 2012, 2013).

Nesfatin-1 is an 82 amino acid peptide derived from the precursor nucleobindin2. It was identified by Oh *et al.*, 2006 and named based on its biological action to reduce the dark-phase food intake and weight gain in rats [nucleobindin2 (NUCB2)-encoded satiety- and fat-influencing protein]. NUCB2/nesfatin-1 immunoreactivity is found in several hypothalamic nuclei and also in other CNS regions such as insular cortex, central amygdaloid nucleus, cerebellum and spinal cord (Goebel *et al.*, 2009). Nesfatin-1 immunopositive

neurons co-localize in forebrain and hindbrain nuclei with a number of peptidergic transmitters regulating food intake: POMC, CART, a-MSH, MCH, oxytocin, NPY (Stengel & Tache, 2010). Central injections of Nefastin-1 into the lateral ventricles of locally into the PVN reduced the dark phase food intake in rats by reducing meal size and increase inter-meal intervals (Goebel *et al.*, 2011). However, no effects were observed in food consumption during the light phase in overnight fasted rats with the same treatment (Stengel *et al.*, 2009) suggesting lower efficacy of nesfatin-1 outside physiological feeding period. In addition to the effects on food ingestion, chronic central infusions of nesfatin-1 reduced, while NUCB2 antisense oligonucleotide increased, body weight gain in rats (Oh *et al.*, 2006).

The appetite-stimulating effects of marijuana (*Cannabis sativa*) are known for a long time by the report of its users to promote strong cravings for and an intensification of the sensory and hedonic properties of food (Kirkham, 2009). Today is well known that the endogenous cannabinoid system might regulate energy balance and food intake at several functional levels, both in the brain, including the limbic system (for hedonic evaluation of food), hypothalamus and hindbrain (integrative functions), and also in the periphery, as in adipose tissue, skeletal muscle, liver and gastrointestinal tract (reviewed in Matias & Di Marzo, 2007; Di Marzo *et al.*, 2009; Silvestri *et al.*, 2011). Endocannabinoid levels vary in the hypothalamus and limbic forebrain according with the nutritional status, with levels being the highest with food deprivation and lowest during food consumption (Kirkham *et al.*, 2002). There is substantial evidence that phytocannabinoids (cannabis constituents) and endocannabinoids (anandamide and 2-arachidonoylglicerol) stimulate appetite and can promote body weight gain in animals and humans, and that these effects are mediated via cannabionoid CB1 receptors. Conversely, CB1 receptor antagonists has been shown to reduce food intake (reviewed in Bellocchio *et al.*, 2006; Gaetani *et al.*, 2008). Moreover, CB1-KO mice show reduced caloric intake and decreased body weight (Poncelet *et al.*, 2003). Such findings led to the development in the 1990s of rimonabant, a CB1 receptor antagonist that suppressed appetite and weight gain in animals and, in four major clinical trials, has been found to produce important weight loss (Despres *et al.*, 2005; Van Gaal *et al.*, 2005; Pi-Sunyer *et al.*, 2006; Scheen *et al.*, 2006). However, growing reports of serious psychiatric problems such as anxiety, depression and suicide led to suspension of marketing authorizations in October

2008. This fact hampered the interest in CB1-receptor-antagonist-based anti-obesity drugs and let to the termination of several drug development programs including those for taranabant, otenabant, surinabant and ibipinabant (Rodgers *et al.*, 2012).

Great part of the evidence supporting the involvement of monoaminergic neuronal system in controlling eating behavior came from clinics, as demonstrated that drugs stimulating monoaminergic transmission suppress hunger and food intake (reviwed in Nelson & Gehlert, 2006). Centrally acting sympathomimetics such as amphetamine, phentermine and diethylpropion were among the earliest pharmacological agents used for weight loss. They were very popular in the 1950s and 1960s, but growing concerns about cardiovascular risk and abuse potential led to a marked decline in their use in the early 1970s and withdraw from market in the 1990s (Rodgers *et al.*, 2012). Generally all stimulants (or its active metabolites) are actively transported by the monoamine transporters, acting as competitive inhibitors of the uptake of the native neurotransmitters. These drugs differed for its selectivity for the monoamine uptake. Amphetamine has broad effects on monoamine uptake but appears to be a little bit most potent at norepinephrine (NE) followed closely by dopamine (DA) (Rothman *et al.*, 2001). Phentermine and N-dealkylated-diethylpropion, the diethylpropione active metabolite, are nearly 10 times more potent into NE than in DA uptake. The relatively greater effect on NE systems than DA may explain the apparent lower abuse liability than the amphetamines (Yu *et al.*, 2000; Nelson & Gehlert, 2006). Fenfluramine was approved by FDA for the short-term treatment of obesity in 1973. *In vivo* fenfluramine is extensively metabolized to norfenfluramine and both increases monoamine release with higher potency to serotonin (5-HT) (Rothman *et al.*, 2003). Unlike fenfluramine, norfenfluramine is a potent agonist at the 5-HT2C receptor subtype. An accumulation of data in animals and humans has suggested that activation of the 5-HT2C receptor by norfenfluramine is responsible for most of the antiobesity effects of fenfluramine in humans (Bickerdike, 2003; Vickers & Dourish, 2004). However, reports of cardiac valvulopathy (Connolly *et al.*, 1997) led the manufacturers to withdraw fenfluramine from the market. Sibutramine, a dual NE and 5-HT uptake inhibitor was originally evaluated in the 1980s as a potential antidepressant, efforts were redirected toward development as an anorectic agent as a result of the serendipitous finding that it produced

weight loss, particularly in obese, depressed patients (Luque & Rey, 2002). It was introduced to clinical practice in the late 1990s but the results in weight loss were very modest. Some important adverse effects, as the cardiovascular risk, emerged as a result of a post-marketing clinical trial (James *et al.*, 2010) and result in the suspension of marketing authorizations (Rodgers *et al.*, 2012).

Peripheral signals controlling food intake

Gastric satiation signals arise primarily from mechanical distension while intestinal signals derive largely from chemical effects of nutrients (Cummings & Overduin, 2007). Mediators of intestinal satiation include a number of gut peptides that are secreted from enteroendocrine cells in response to ingested food. These messengers diffuse through intestinal fluids to activate nearby extrinsic sensory fibres (vagal and spinal afferents) and/or enter the bloodstream to function as hormones (Park & Camilleri, 2005). Satiation signals from intestine are generally short-lasting, but they can interact at several levels with long-acting adiposity hormones involved in body-weight regulation (Cummings & Overduin, 2007; Capasso & Izzo, 2008).

The first gut hormone found to be implicated in appetite control was cholecystokinin (CCK) (Gibbs *et al.*, 1973). This hormone is secreted post-prandially from the L cells of the small intestine into circulation with a plasma half-life of few minutes (Liddle *et al.*, 1985). Two types of CCK receptors were identified: CCK1 receptors, present in peripheral tissues such as the pancreas, gallbladder and on vagal afferent nerve fibres innervating the gut (Moran & Kinzig, 2004) and also within the CNS in the DMH, NTS and AP (Moran *et al.*, 1986). CCK2 receptor has a different distribution and is found in gastric mucosa, vagal afferents, hypothalamic nuclei and cortex (Moran *et al.*, 1986; Sam *et al.*, 2012). Peripheral administration of CCK in rodents results in a dose dependent reduction of food intake, decreasing both meal size and duration and is associated with expression of post-prandial satiety behaviours such as increased groomings and decreased locomotor activity (Antin *et al.*, 1975). In humans, intravenous administration of physiological doses of CCK reduces food intake and increases the perception of fullness (Lieverse *et al.*, 1995)

Ghrelin is the only known orexigenic gut hormone. Circulating ghrelin levels rise preprandially and fall rapidly in the post prandial period (Cummings *et al.*,

2001). Fasting plasma levels are reported to be high in patients with anorexia nervosa (Otto *et al.*, 2001) and subjects with diet-induced weight loss (Cummings *et al.*, 2002). In contrast, ghrelin levels are low in obese subjects (Shiiya *et al.*, 2002). Central and peripheral ghrelin administration increases food intake and weight gain in rodents (Tschop *et al.*, 2000; Wren *et al.*, 2000; Lawrence *et al.*, 2002). Moreover, intravenous ghrelin administration also stimulates gastric acid secretion and motility in rats (Masuda *et al.*, 2000). Peripheral administration of ghrelin increases c-Fos expression in NPY/AgRP neurons in the ARC nucleus (Wang *et al.*, 2002) and ablation of these neurons completely abolishes the orexigenic effect of ghrelin (Chen *et al.*, 2004). When given centrally ghrelin also stimulates c-Fos expression in other nuclei involved in appetite control including the PVN, DMH, LH as we as in the NTS and AP in the brainstem (Lawrence *et al.*, 2002).

Glucagon-like peptide-1 (GLP-1) is a 30 amino acid peptide resulting from cleavage of the preproglucacon precursor molecule. The two bioactive forms $GLP1_{7\text{-}37}$ and $GLP1_{7\text{-}36}$ amide are released into the circulation from L cells in the intestine (Herrmann *et al.*, 1995). Both GLP-1 and the GLP-1 receptor were found in the brain especially in the hypothalamic nuclei (Larsen *et al.*, 1997). Acute peripheral and central GLP-1 injections reduce food intake in rats (Turton *et al.*, 1996; Tang-Christensen *et al.*, 2001) and chronic administration reduces weight gain (Meeran *et al.*, 1999). Peripheral administration of GLP-1 in rats leads to increased c-Fos expression in the ARC (Abbott *et al.*, 2005), intracerebroventricular administration results in increased c-Fos expression in the PVN, NTS and AP (Larsen *et al.*, 1997). Moreover, GLP-1 delays gastric empting (Schirra *et al.*, 2006) and increases satiety (Punjabi *et al.*, 2011). These effects are thought to be mediated through vagal transmission because they are abolished in vagotomised animals (Imeryuz *et al.*, 1997; Abbott *et al.*, 2005). In addition to its anorexic effect, GLP-1 is an important incretine, stimulating glucose dependent insulin release (MacDonald *et al.*, 2002) and inhibiting glucagon secretion (Willms *et al.*, 1996) resulting in a reduction of blood glucose levels after a meal suggesting its utility in diabetes treatment (Sam *et al.*, 2012). However, GLP-1 use is limited by its short half-life due the rapid degradation mediated by the enzyme dipeptidyl peptidase (DDP-IV) for this reason long acting GLP-1 mimetics, like exenatide and liraglutide, have been developed as new treatments for type 2

diabetes (Joy *et al.*, 2005). In phase III clinical trials, exenatide reduced body weight in diabetics patients (Buse *et al.*, 2004; DeFronzo *et al.*, 2005; Kendall *et al.*, 2005). Liraglutide caused sustained weight loss in both diabetics (Astrup *et al.*, 2012) and non-diabetic (Lean *et al.*, 2013) obese patients, suggesting the efficacy of these compounds for the treatment of obesity.

Leptin is the product of the *ob* gene, and it is predominantly secreted by adipocytes with circulating levels proportional to fat mass (Considine *et al.*, 1996). Leptin is transported across the blood brain barrier by a saturable transporter system (Banks *et al.*, 1996). In the ARC nucleus, leptin by activating its receptors (LepRb) inhibits NPY/AgRP neurons and activates POMC/CART neurons (Schwartz *et al.*, 2000) resulting in reduced food intake and increased energy expenditure (Pelleymounter *et al.*, 1995). The *db/db* mouse, carries an inactivating mutation in the LepRb receptor and has as obese phenotype (Chen *et al.*, 1996) and also leptin-deficient *ob/ob* mice exhibit hyperphagia and obesity, which can be reversed by leptin administration (Halaas *et al.*, 1995). Subcutaneous administration of recombinant leptin reduces fat mass, hyperinsulinaemia and hyperlipidaemia in obese children with congenital leptin deficiency (Farooqi *et al.*, 2002). However, obese individuals often have high leptin levels which results in a failure to respond to exogenous leptin treatment. This resistance, that could be result from reduced LepRb signal transduction or impaired ability to cross the blood brain barrier (Munzberg, 2010), severely limits the therapeutic utility of leptin for obesity treatment.

Oleoylethanolamide: a lipid-derived satiety factor

The infusion of lipid emulsions into the small intestine has been demonstrated to rapidly and potently suppress food intake and mammalian species, including humans (Castiglione *et al.*, 1998; French *et al.*, 2000). Local release of CCK and serotonin has been implicated in these responses (Schwartz, 2011a). New evidences suggest, however, an important role for a different class of signalling molecules, the fatty acid ethanolamides or FAEs (Gaetani *et al.*, 2008). FAEs were isolated from animal tissues more than 50 years ago (Bachur *et al.*, 1965). These days is known that FAEs participate in a variety of physiological and pathological processes. Indeed, palmitoylethanolamide (PEA) has anti-nociceptive and anti-inflamatory properties (Lambert *et al.*, 2002). Anandamide (ANA) the

endogenous ligand of the cannabionoid receptor 1 (CB1) have many biological activities, including increase of food intake (Luchicchi & Pistis, 2012). Another interesting FAE is the oleic acid derivative oleoyletanolamide or OEA. Biological functions of OEA, such as anorexigenic and body fat loss properties have been extensively studied over the past decade (reviewed in (Thabuis *et al.*, 2008; Piomelli, 2013).

The biosynthesis pathway described for OEA suggests that the enterocytes use food-derived oleic acid and phosphatidylethanolamine to generate, through the action of N-acyltransferase (NAT), the phospholipid *N*-oleoyl-phosphatidylethanolamine (NOPE), which is cleaved by *N*-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD) originating OEA (Fu *et al.*, 2007). The hydrolysis of OEA into oleic acid and ethanolamine is considered to be the primary mechanism through which the biological actions of this lipid mediator are terminated. Two intracellular amidases have been implicated in this process: fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine acid amidase (NAAA). The first is highly expressed in the central nervous system, liver and small intestine (Katayama *et al.*, 1997). The second is highly expressed in macrophages and pharmacological evidence suggests that NAAA regulates tissue FAE levels during inflammation (Solorzano *et al.*, 2009), but its role in small-intestinal OEA degradation remains to be defined.

Levels of OEA change according to the nutrient status: they are lower in food-deprived than in free-feeding animals and increase upon refeeding (Rodriguez de Fonseca *et al.*, 2001; Astarita *et al.*, 2006; Petersen *et al.*, 2006). Three simultaneous events are responsible for the feeding-induced OEA formation: enhanced production of NAPEs, activation of NAPE-PLD and inhibition of FAAH activity (Fu *et al.*, 2007). Experiments in which individual nutrients were infused separately into the duodenum of catheterized rats showed that fat is a potent stimulus for jejunal OEA production, whereas sugar and protein have no such effect. More detailed investigation revealed that intraduodenal infusions of oleic acid, but not palmitic acid stimulate mucosal cells to generate OEA (Schwartz *et al.*, 2008).

Administration of OEA through the intraperitoneal or oral rout causes a long-lasting inhibition of food intake in rats and mice (Rodriguez de Fonseca *et al.*, 2001; Fu *et al.*, 2003; Gaetani *et al.*, 2003; Nielsen *et al.*, 2004; Oveisi *et al.*, 2004;

Proulx *et al.*, 2005). Detailed analysis of the meal pattern in free-feeding mice revealed that OEA increases feeding latency and decreases meal frequency without changes in meal size (Gaetani *et al.*, 2003) suggesting that OEA induces a satiety state. Interestingly, prolonged exposure to dietary fat reduced small-intestinal FAE levels in rats (Artmann *et al.*, 2008; Diep *et al.*, 2011) raising the possibility that diets rich in fat might promote overeating, at least in part, by suppressing the satiating effects of gut-derived OEA (Piomelli, 2013).

Several lines of evidence support the idea that OEA causes satiety by activation of peroxisome proliferator-activated receptor-alpha (PPAR-alpha) a nuclear receptor linked to the regulation of absorption, storage and utilization of dietary fat (Schupp & Lazar, 2010; Varga *et al.*, 2011). *In vitro* studies demonstrated that OEA binds with relatively high affinity (K_D = 37.4 nM) the ligand-binding domain of PPAR-alpha and stimulated transcriptional activity mediated by this receptor (EC_{50} = 120 nM) (Fu *et al.*, 2003). Interesting in this regard is that after feeding, endogenous levels of OEA in gut mucosa rise to concentrations nearly 300 nM (Fu *et al.*, 2007) that are sufficient to activate PPAR-alpha. Moreover, OEA hypophagic effect was mimicked by GW7647 and WY14643, two PPAR-alpha agonists, and was abolished in mice lacking PPAR-alpha (Fu *et al.*, 2003). OEA also activates two other receptors: the capsaicin receptor TRPV1 (66,67) and GPR119 (68), a G protein-coupled receptor that recognizes a broad panel of lipid molecules (69). Nevertheless, the observation that genetic deletion of TRPV1 or GPR119 in mice does not alter the anorexic effects of OEA strongly argues against a direct involvement of these receptors in OEA-induced satiety (Lo Verme *et al.*, 2005; Lan *et al.*, 2009).

When OEA was administrated in pH-protective capsules (releasing OEA at pH = 6) comparable effect was observed at a fourfold lower dose as compared to OEA given by gavage (Nielsen *et al.*, 2004; Oveisi *et al.*, 2004). Moreover, effects of OEA capsules were significantly different from controls 5h after administration, which corresponds to the time for the capsules to go from the stomach to the small intestine, confirming that OEA acting locally within the small intestine (Nielsen *et al.*, 2004).

Although OEA infusion into the brain ventricles have no effect in food intake, surgical disconnection of the vagus nerve prevents the hypophagic effect induced by systemic OEA injections and similar failure was observed in rats

treated with neurotoxic doses of capsaicin, which deprive the animals of peripheral vagal and non-vagal sensory fibres (Rodriguez de Fonseca *et al.*, 2001) suggesting participation of central mechanisms in OEA-induced hypophagia. Indeed, peripherally administered OEA selectively stimulates c-Fos expression in brain areas involved in the central control of satiety and energy balance such as the NST in the brainstem and the PVN and SON nuclei in the hypothalamus (Rodriguez de Fonseca *et al.*, 2001; Gaetani *et al.*, 2010). In both hypothalamic nuclei, c-Fos was increased in neurons expressing oxytocin, whose activation is paralleled by increased peptide immunoreactivity in the posterior pituitary gland and elevated circulating oxytocin levels, thus suggesting a stimulatory effect of OEA on oxytocin neurosecretion (Gaetani *et al.*, 2010; Romano *et al.*, 2013a). No effect is observed on vasopressin expressing neurons or circulating vasopressin levels (Gaetani *et al.*, 2010; Serrano *et al.*, 2011). Moreover, pharmacological blockade of central oxytocin receptors abrogates the hypophagic effects of OEA without blocking c-Fos activation on NTS (Gaetani *et al.*, 2010). These observations suggest that PVN involvement is downstream to NTS activation. The same research group recently demonstrated that lesions of the noradrenergic neurons in the NTS that project to the PVN prevented the OEA's effects on food intake, on c-Fos and oxytocin expression in the PVN and on oxytocin immunoreactivity on the posterior pituitary gland (Romano *et al.*, 2013b). In line with previous demonstrations that peripheral administered OEA increased hypothalamic noradrenaline concentration (Serrano *et al.*, 2011) these findings support the hypothesis that noradrenergic NTS-PVN projections are involved in the activation of the hypothalamic oxytocinergic system, which mediates OEA's pro-satiety action.

Noradrenergic projections connect the NTS also with the basolateral complex of the amygdala (BLA), and are a part of a neural circuit that is crucially implicated in the consolidation of emotional memories (McGaugh, 2000). Using two distinct experimental paradigms in rats – inhibitory avoidance and the Morris water maze – (Campolongo *et al.*, 2009) found that i.p. injection of OEA after training strongly increased memory retention in these tasks. Such effect was blocked by lidocaine infusions into the NST or the beta-adrenergic antagonist propranolol into the BLA, suggesting that peripheral OEA reach the CNS via the afferent vagus and improves memory consolidation by stimulating noradrenergic

activity in the BLA. It appears, therefore, that OEA signalling in the gut initiates an integrated response in which satiety induced by a rich-fat meal coincides temporally with enhanced encoding of information about the spatial and emotional context in which the meal was consumed, that could be rationalized as an adaptative strategy to improve foraging (Piomelli, 2013).

Brain histamineric system in feeding and energy metabolism.

Histamine containing neurons constitute a major wake-promoting system (Lin *et al.*, 2011) and wakefulness is a prerequisite for learning and memorizing salient events. Moreover, there is ample evidence that the histaminergic system affects cognitive function *per se* (Kohler *et al.*, 2011). These observations are relevant for feeding behaviour, both for food searching and food consumption as the ability to remember the context associated with food availability or the palatability of the food eaten provides a clear adaptive advantage to animal foraging (Munari & Passani, 2011).

Early studies indicated an inverse relationship between brain histaminergic activity and feeding. It was found that histamine injected i.c.v. produces a long-term suppression of feeding activity in cats (Clineschmidt & Lotti, 1973). Similarly, the administration of histamine into the suprachiasmatic nucleus (Itowi *et al.*, 1988) or in the lateral ventricle (Lecklin & Tuomisto, 1998) also decreased food intake in rats. Other manipulations increasing brain histaminergic availability such as systemic administration of the precursor L-histidine (Sheiner *et al.*, 1985; Vaziri *et al.*, 1997), or the HNMT inhibitor metoprine (Lecklin *et al.*, 1995), also suppressed food intake. Conversely, restriction of neuronal histamine synthesis by alpha-FMH, increases significantly food consumption (Orthen-Gambill & Salomon, 1992; Ookuma *et al.*, 1993; Tuomisto *et al.*, 1994).

Histamine suppression of food intake appears to be mediated via activation of H1Rs. Centrally administered H1R agonists suppressed in rats (Lecklin *et al.*, 1998; Lecklin & Tuomisto, 1998), whereas injection of H1R antagonist elicited (Sakata *et al.*, 1988a) food intake in rats. The effects of H1R antagonist seems to be site specific, since microinfusions of these compounds locally into the VMH or PVN but not into the LH or DMH elicit feeding responses and increases both meal size and duration (Sakata *et al.*, 1988b; Sakata *et al.*, 1988c; Fukagawa *et al.*, 1989; Ookuma *et al.*, 1989). H1R agonist- induced increased in food intake was

prevented by alpha-FMH injections (Sakata *et al.*, 1988b). In keeping with the pharmacological manipulations of the H1R, genetically modified H1R-KO mice exhibit an increase in daily food consumption and visceral adiposity (Masaki *et al.*, 2004). H2R seems not to be involved in feeding control as both agonists (Lecklin *et al.*, 1998) and antagonists (Sakata *et al.*, 1988b; Sakata *et al.*, 1988c) had no effect on food intake.

Histamine seems to contribute from both appetitive and consummatory phases of feeding behaviour. Valdes *et al.*, 2010 showed an increase in histamine release in the posterior hypothalamic area when hungry rats were trying to open a mesh container filled with enticing food. Other studies suggest that histaminergic activity in the rat hypothalamus increases during feeding as demonstrated by the transient and significant increase in histamine concentration when 24-h fasted rats were fed (Itoh *et al.*, 1991; Ishizuka & Yamatodani, 2012).

Brain histamine affects both sides of the energy balance: by decreasing intake and increasing expenditure. One of the markers of energy expenditure are *Uncoupling proteins* as uncoupling protein 1 (Ucp1) and uncoupling protein 3 (Ucp3) present in brown and white adipose tissues, respectively. Central infusions of histamine caused an upregulation in the expression of Ucp1 and Ucp3 mRNA in normal but not in H1R-KO mice (Masaki *et al.*, 2001a; b). Central histamine injection augmented lipolysis in rat adipose tissue, and thioperamide mimicked histamine action. Intraperitoneal pretreatment with propranolol, a beta-adrenoceptor antagonist, abolished the thioperamide-induced lipolytic action suggesting that the effect is mediated by sympathetic nerves that innervate adipose tissue (Tsuda *et al.*, 2002). TMN neurons are also essential for the increase in temperature during a motivated behaviour (Valdes *et al.*, 2010) and also for increasing arousal, leading to increased motor activity and, altogether enhancing energy spending (Passani *et al.*, 2011).

***The relationship between neural histamine and peptides that control feeding behaviour*.**

Several peptides such as orexin, leptin, GLP-1, thyrotropin releasing hormone (TRH), nesfatin-1 require, at least in part, the histaminergic signalling in the brain to exert their orexigenic or anorexigenic effects (Munari & Passani, 2011).

Histamine and orexin neurons exert different, but complementary, control on wakefulness, the former being more important for aspect of consciousness and cognitive functions, whereas the latter are involved primarily in behavioural arousal including muscle tone, locomotion and emotional reactions (Anaclet *et al.*, 2009). There is a close and reciprocal anatomical connection between histaminergic and orexinergic neurons (REF). *In vitro*, orexin strongly excites TMN neurons (Eriksson *et al.*, 2001). Perfusion of orexin A into TMN increases wakefulness (Huang *et al.*, 2001) and stimulates food intake in rats (Jorgensen *et al.*, 2005), effects that are dependent of an intact histaminergic neuronal system and seems to involve a mechanism mediated by H1R.

The interaction of leptin and the histaminergic system has been well investigated by several researchers. Histamine release was shown to significantly increase when leptin was administered intraperitoneally to rats (Morimoto *et al.*, 1999). Accordingly, leptin-induced suppression of food intake was significantly attenuated in alpha-FMH-treated (Yoshimatsu *et al.*, 1999; Toftegaard *et al.*, 2003) and H1R-KO mice (Morimoto *et al.*, 1999; Masaki *et al.*, 2001b; Mollet *et al.*, 2001). Chronic infusion of histamine into the third ventricle prevents the development of obesity in hyperleptinemic animals: both *db/db* mice and diet-induced obese (DIO) mice (Masaki *et al.*, 2001a).

The hypophagic effect induced by GLP-1 seems to be mediated, in part, by the neuronal histaminergic system. Central infusions with GLP-1 augmented the histamine turnover in the hypothalamus and induced hypophagic effect which was partially attenuated in histamine-deprived rats (Gotoh *et al.*, 2005)

TRH is secreted by neurones in the PVN. It suppresses food intake, activates the TMN neurones (Parmentier *et al.*, 2009) and increases histamine turnover in the hypothalamus (Gotoh *et al.*, 2007). In food deprived H1R-KO mice and acute histamine-depleted rats, TRH-induced suppression of feeding is significantly attenuated (Gotoh *et al.*, 2007).

Also the effect of the recently discovered anorexic neuropeptide Nesfatin-1 was partially attenuated in rats administered with alpha-FMH and in H1KO mice. Nesfatin-1 central injection increased histamine turnover, whereas histamine centrally injected increased nesfatin-1expression in the hypothalamus. Immunohistochemical analysis revealed H1R expression on nesfatin-1 neurons in the PVN suggesting this nucleus as a site where the interaction between these

systems occur (Gotoh *et al.*, 2013)

All together these observations further prove the complexity of the histaminergic system as a regulator of food intake and energy metabolism, as both orexigenic and anorexigenic effects of endogenous molecules appear to require the integrity of the central histaminergic system.

H3 receptors and feeding behaviour

Although central H1R seems to be a valid target to control food intake, to date, selective H1R-agonists which can penetrate to the brain and devoid of peripheral action are unavailable. Therefore the use of compounds that enhance histamine release form nerve terminals, such as H3R antagonists/inverse agonist may afford an effective therapeutic alternative. However, the role of H3 receptor ligands in the treatment of obesity is still unclear as controversial findings from different studies were described.

Most experimental observations in rodents seem to agree that blockade of brain H3R is beneficial in decreasing energy intake, body weight and plasma triglycerides (reviewed in (Hancock & Brune, 2005; Passani *et al.*, 2011). Indeed, experiments demonstrated that H3R antagonists increase histamine release from the hypothalamus and reduce energy intake in normal and leptin-resistant mice with diet induced obesity (Ishizuka *et al.*, 2008). In addition, administration of H1R receptor antagonists attenuates the feeding suppression induced by H3R antagonists (Hancock *et al.*, 2005). H3R agonists reduce satiety induced by amylin (Lutz *et al.*, 1996) or bombesin (Kent *et al.*, 1997) and CCK (Attoub *et al.*, 2001), whereas H3R antagonists attenuate the orexigenic effect of NPY (Itoh *et al.*, 1998; 1999) and enhanced CCK-induce satiety (Attoub *et al.*, 2001).

Not all data, though, support an appetite-suppressant effect of H3R blockade.In diet-induced obese mice, an H3R agonist suppresses food intake and decreases body weight, presumably with a mechanism independent of histamine release modulation (Yoshimoto *et al.*, 2006). Furthermore, H3R-KO mice manifest disrupted regulation of body weight, energy expenditure and food intake resulting in obese hyperphagic mice with reduced energy expenditure, which resembles the phenotype of H1R-KO (Takahashi *et al.*, 2002). Considering these data, it is clear that the effects of H3R modulators on food consumption and metabolism are more complex and not only mediated by histamine release, but they are regulated

through a variety of receptors and neurotransmitters and may be responsible for the discrepancies described above.

An interesting application of the H3R could be in attenuating antipsychotic-induced weight gain. A combined treatment of betahistine (a H1R agonist and H3R receptor antagonist) reduced food intake and the body weight gain induced by olanzapine in rats (Deng *et al.*, 2012). The administration of the same combination (olanzapine/betahistine) to schizophrenic patients for 6 weeks produced a significant weight gain in the first 2 weeks with no additional increase of weigh up to the end of trial (Poyurovsky *et al.*, 2005). Unfortunately, in this study there was no placebo group making difficult the interpretation of this result. Recently the same group reported the result form a double-blind placebo-controlled clinical trial in which it was observed that patients treated with a combination of olanzapine/reboxetine + betahistine gained significantly less weight than those treated with olanzapine/placebo (Amrami-Weizman *et al.*, 2013). The weight-attenuating effect of the combination betahistine+reboxetine was twofold larger than the weight-attenuating effect previously demonstrated with reboxetine alone (Poyurovsky *et al.*, 2007). However, it is important to note that patients enrolled in clinical trials to test the efficacy of other H3R antagonists (pitolisant or MK-0249) in narcolepsy or attention-deficit hyperactivity disorder did not report significant weight changes. Nonetheless, these compounds may turn out to be effective in test evaluating specifically eating disorders. In this regard a multicenter, randomized, placebo-controlled phase II clinical trial to evaluate the efficacy of SCH497079 on weight in obese and overweight subjects was recently completed by the results are not yet disclosed (www.clinicaltriasl.gov). Given the substantial differences of the preclinical outcome and the discrepancies in clinical trials, considerable experimental effort remains necessary to prove the so far unclear concept of H3R antagonists in the treatment of obesity and weight gain (Passani *et al.*, 2011).

AIMS

Several endogenous molecules contribute to build a complex network of neural and hormonal signals to align food intake and energy expenditure. The endogenous lipid messenger OEA engages sensory fibers of the vagus nerve that project centrally activating central oxytocin transmission in the PVN and SON

inhibiting food intake. However, it is not known whether other neurotransmitters contribute to its anorexic effects. We hypothesized that peripherally administered OEA engages histamine signalling in the brain. Using a combination of behavioural, neurochemical and immunological techniques we evaluated the effects of manipulations of the central histaminergic system in the anorexiant effect and neurochemical modifications induced by OEA systemic administration. Our findings revealed that the anorexic effects of OEA are blunted in brain histamine deficient mice, and we suggest that the hypophagic effects of histamine and OEA converge on oxytocic neurons in the hypothalamic PVN.

METHODS

Animals

Male CD1 outbreed mice (25-35 g; Harlan, Italy) and histidine decarboxylase knock out (HDC-KO) mice and wild type (WT) littermates (background sv129), breeded in the Centro Sabulazione Animali di Laboratorio (CeSAL), Universitá di Firenze. Animals were housed in an humidity and temperature-controlled room (22 - 24 °C) with a 12:12-h light-dark cycle (lights start at 07:00 AM) with standard diet (4RF21; Mucedola s.r.l., Milan, Italy) and freely available water. HDC-KO and WT were used at 2-3 months of age (25-35 g). Mice were handled for one week before experiments. Housing, animal maintenance and all experiments were conducted in accordance with the Council Directive of the European Community (86/609/EEC) of the Italian D.L. 116 (1992), NIH guidelines on animal care and approved by veterinarian supervision.

Drugs

OEA (Tocris Bioscience, Bristol, UK) was dissolved in saline/polyethylene glycol/Tween80 (90/5/5, v/v). ABT-239 and αFMH were synthesized at Abbott Laboratories (Abbott Park, IL 60064, USA) and dissolved in physiological saline. All other reagents and solvents were of HPLC grade or the highest grade available (Sigma, UK).

Evaluation of cumulative food consumption

Mice were tested during the light-on period (8:30 a.m.) after 12h food-deprivation while water remained available. After the treatments (Figure III.1), a weighed

amount of standard chow pellets was placed in the food rack, and food consumption evaluated as the difference in weight between that of initially provided food and that left in the rack, including spillage in the cage. Food consumption was measured 15, 30, 45, 60 min, and 2, 4, 6, 8, 10, 24 hours after food presentation.

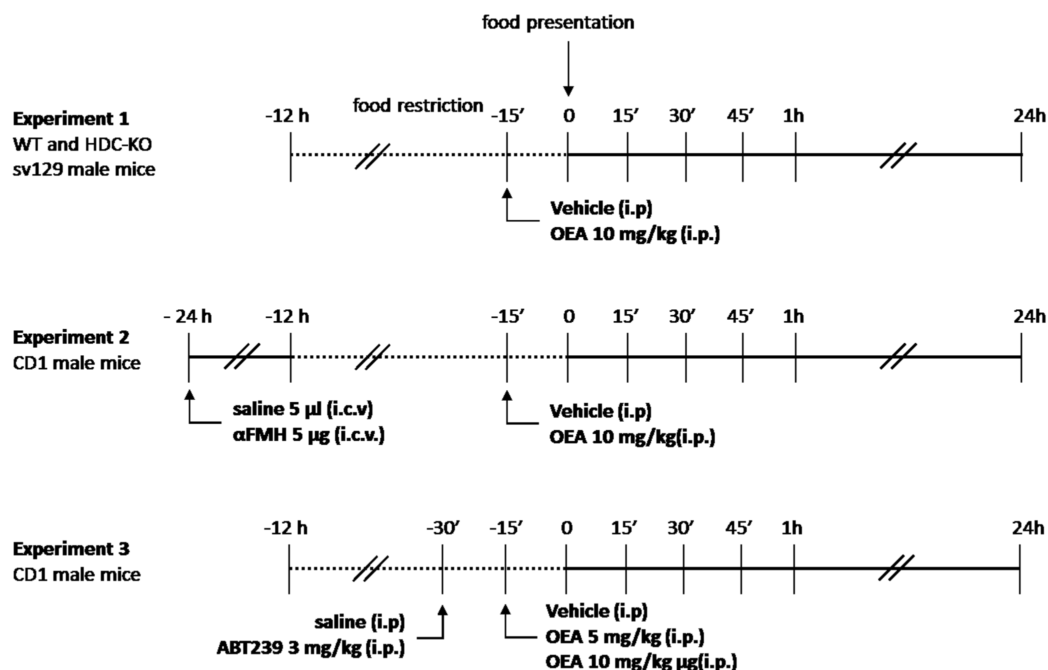

Figure III.1. Schematic representation showing the experimental protocols used to evaluate the effects of manipulations of central histaminergic system on the OEA anorexiant effect. Dotted line represents the food restriction period (12 h). After the different treatments and then a weighed amount of standard chow pellets was placed in the food rack and food consumption was measured at pre-determined time intervals.

Microdialysis experiments

CD1 mice were placed in a stereotaxic frame under deep anaesthesia with isoflurane (5% in oxygen) and a guide cannula (CMA 7, CMA Microdialysis, Sweden) was inserted in the pre-frontal cortex using the following coordinates according to (Paxinos & Franklin, 2001) AP = +2.1, L = +1.0 DV= +1.5. The cannula was fixed to the skull using dental cement. Mice treated with α-FMH were injected into the lateral ventricle during surgery (coordinates AP, -0.3; L, -1; DV, 2.4). After surgery mice were singularly housed to recover for 24-48 hours. On the experiment day, the dialysis membrane (2 mm, cut off 6 kD, CMA 7 MD, CMA Microdialysis, Sweden) replaced the obstructor. The probe was perfused with Ringer's solution (NaCl 147 mM, $CaCl_2$ 2.2 mM, KCl 4.0 mM) for 2h stabilization

(flux=1 µL/min). After collection of three 30-min baseline samples the different treatments were systemically administered. In the study aiming to evaluate the effects of food-deprivation on histamine release, the animals were fasted 12 hours before the experiments (Figure III.2). Experiments were performed between 9:00h and 16:00h. To prevent histamine degradation, 1.5 µL of 5 mM HCl was added to each sample. Dialysates were immediately frozen and stored at -80 °C until analysis. Histamine determination and histological verification of the correct probe position were performed as described in chapter I.

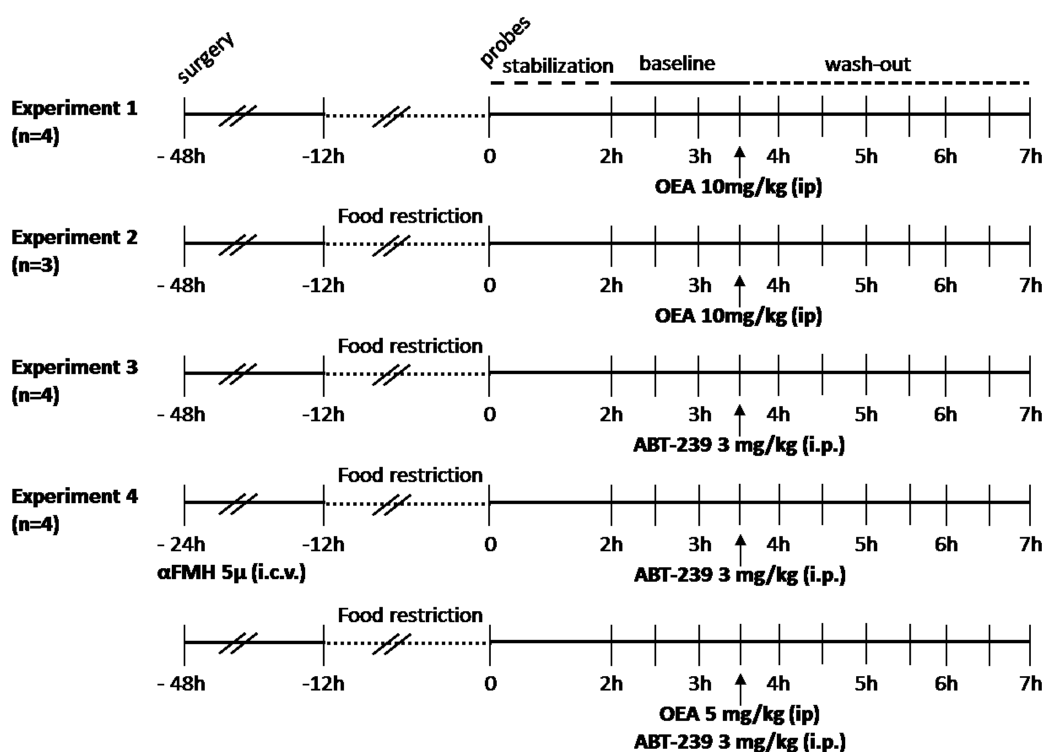

Figure III.2. Schematic diagram showing experimental protocols used in the microdialysis study. Mice were implanted with one probe into the prefrontal cortex to measure neurotransmitter release. The traced line represents the period of food restriction (12 h).

Immunohistochemistry

HDC-KO and WT littermates housed 4/cage were maintained on standard chow diet and food-deprived for 12h (between 20:00 and 8:00, water remained available) before i.p. administration of OEA (10 mg/kg) or saline. Two hours after injections, mice were deeply anaesthetized with chloral hydrate and perfused transcardially with cold physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were postfixed in the same solution overnight (4 ºC), and cryoprotected in 30% sucrose in PB. The methodology used

for c-Fos immunostaining was the same described in the chapter I. Atlas coordinates relative to bregma (Franklin and Paxinos, 2007) for the sections analysed were: from +1.70 to +1.34 for the infralimbic cortex (IL Cx), from +1.18 to +0.86 for the NAcc, from -1.46 to -1.70 for the VMH and from −0.82 to -0.94 for the PVN. Statistics were calculated on the average values from three to six sections of individual animals. Thus, sample size and statistics were based on number of mice.

Immunofuorescence

For double-labelling experiments, sections were preincubated with 2% normal goat and donkey sera (NDS, NGS Jackson Immunoresearch, West Grove, PA, USA) in PB-TX for 1h and then incubated in a cocktail of c-Fos (1:3000) and mouse oxytocin (1:1000; Millipore, Temecula, CA) primary antibodies in PB-TX, 1% NGS, 1% NDS overnight at 4 ºC. Sections were then incubated in Cy3-conjugated donkey anti-rabbit IgG (1:400; Jackson Immunoresearch, West Grove, PA) for 2h at room temperature and then in AlexaFluor 488-conjugated, goat anti-mouse IgG, (1:300; Molecular Probes, Eugene, OR, USA) for 2h at room temperature. Sections were mounted on glass slides, coverslipped with anti-fading medium (Vectastain Vector Laboratories, Burlingame CA USA) and observed with a Bio-Rad MCR 1024 ES confocal laser scanning microscope (Bio-Rad, Hercules, CA) equipped with a Krypton/Argon laser source 15 mW for fluorescence measurements as previously described (Giannoni *et al.*, 2009). Single (oxytocin) and double-labelled (oxytocin and c-Fos) neurons were counted bilaterally in at least four sections in each PVN from 3 mice per experimental group using the Image J software (NIH, USA). Statistics were calculated on the average values of individual animals.

Statistical analysis

Statistical analysis was performed using Prism Software (GraphPad Software). Statistical significance of cumulative food consumption and c-Fos expression was determined by two-way ANOVA (time x treatment) with Bonferroni post hoc test and one-way ANOVA with Newman Keuls post hoc test, respectively. Statistical significance of oxytocin immunopositive cells and double labelled PVN neurons was determined by unpaired t-test. Values obtained in microdialysis

experiments are expressed as means ± SEM and analyzed with one-way ANOVA and Bonferroni post-hoc test. For clarity purposes we reported in figures and figure legends only the significant differences vs. the last baseline sample before drug treatment. However, differences were significant versus all baseline samples. The criterion value for all statistical tests was $P<0.05$.

RESULTS

Interaction between brain histamine and OEA on food consumption

To test if the integrity of the histaminergic system contributes to the anorexiant effect of OEA, we used histidine decarboxylase knockout mice (HDC-KO), hence unable to synthesize histamine. OEA was injected i.p. at a dose of 10 mg/kg that induces long lasting appetite suppression (Fu *et al.*, 2003), does not readily enter the CNS (Campolongo *et al.*, 2009) and does not cause taste aversion (Proulx *et al.*, 2005). Controls received equivalent volumes of vehicle. Two-way (treatment x time) ANOVA revealed an overall significant difference between groups [$F_{(time)4,170} = 133.14$, $P<0.0001$; $F_{(treatment)3,170} = 185.64$, $P<0.0001$; $F_{(time\ x\ treatment)12,170} = 12{,}93$, $P<0.001$). OEA caused a profound reduction in total amount of food consumed by WT mice compared to vehicle-treated littermates within the first 60 minutes after injection ($P<0.001$; Figure III.3A). As expected, vehicle-treated HDC-KO mice consumed comparable amounts of food with respect to WT animals [histamine-deficient mice are not hyperphagic, nor obese up to 12 weeks of age (Fulop *et al.*, 2003)], whereas the anorexic effect of OEA was significantly diminished in HDC-KO mice ($P<0.01$ at 45 min; $P<0.001$ at 60 min; Figure III.3A). The effect of OEA was short lived as no difference in food consumption was observed among experimental groups 4 h after OEA injection (not shown).

To exclude the involvement of compensatory mechanisms due to chronic depletion of histamine, we measured food consumption in CD1 mice that received i.c.v. infusions of the HDC suicide inhibitor αFMH (Figure III.3B). Two-way (treatment x time) ANOVA revealed an overall significant difference among groups [$F_{(treatment)3,130} = 68.14$; $P<0.0001$); $F_{(time)4,130} = 51.68$, $P<0.0001$; $F_{(time\ x\ treatment)12,130} = 5{,}5665$, $P<0.001$). Mice from the αFMH/VEH-treated group tended to eat more than SAL/VEH animals, although this effect did not reach statistical significance. OEA induced a profound reduction in the total amount of food consumed by CD1 mice

that received i.c.v. injections of saline. In αFMH-treated mice OEA-induced hypophagia was significantly less prominent than in the SAL/OEA group. The effect reached statistical significance 45 min after OEA injection (P<0.01). The overall trend is very similar to the results obtained with HDC-KO (Figure III.3B).

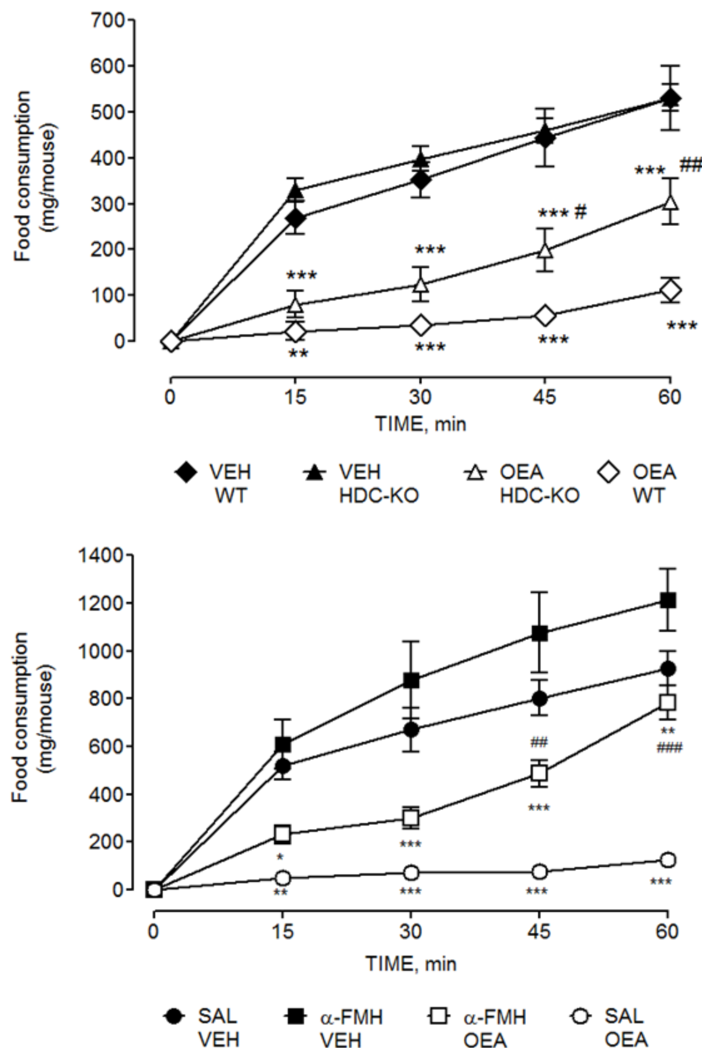

Figure III.3. Brain histamine mediates the appetite-suppressing effects of OEA. (A) Time course of the effect of systemic administrations of OEA (10 mg/Kg, i.p.) or vehicle on cumulative food intake in 12h fasted HDC-KO and WT mice. Each point represents mean cumulative food consumption ± S.E.M of 8-11 mice. ***P<0.001, **P<0.01, *P<0.05 vs respective controls; ###P<0.001, ##P<0.01 vs OEA treated WT mice by Two-way ANOVA and Bonferroni test. (B) Time course of the effect of αFMH administration (5 μg; i.c.v.) or saline on OEA-suppressed food intake in 12h fasted mice. Each point represents mean cumulative food consumption ± S.E.M of 7-8 mice. ***P<0.001, **P<0.01, *P<0.05 vs respective controls; ###P<0.001, ##P<0.01 vs OEA treated αFMH injected mice by Two-way ANOVA and Bonferroni test.

We argued that, if OEA modulates feeding by recruiting the central histamine system, augmenting brain histamine release pharmacologically would

potentiate the anorexic effect of exogenous OEA. We measured food-consumption in food-deprived CD1 mice treated with a combination of ABT-239 (3 mg/kg), an H_3 receptor antagonist that increases histamine release by blocking H3 autoreceptors, and different doses of OEA (5 and 10 mg/kg). As shown in figure III4.A 3 mg/kg ABT-239 decreased significantly food intake in a comparable manner to OEA 5 mg/kg [Two way ANOVA: ($F_{(time)4,128}$ = 519.6, P<0.0001; $F_{(treatment)3,128}$ = 38.98, P<0.0001; $F_{(time\ x\ treatment)12,128}$ = 23, P<0.001)]. Treatment with both compounds determined a further decrease in food intake (P<0.001 at 30 min). Similar results were obtained with 3 mg/kg ABT-239 and 10 mg kg OEA as shown in Figure III4.B [Two way ANOVA: ($F_{(time)4,108}$ = 392.5, P<0.0001; $F_{(treatment)3,104}$ = 31.81, P<0.0001; $F_{(time\ x\ treatment)12,104}$ = 31,81 P<0.001)].

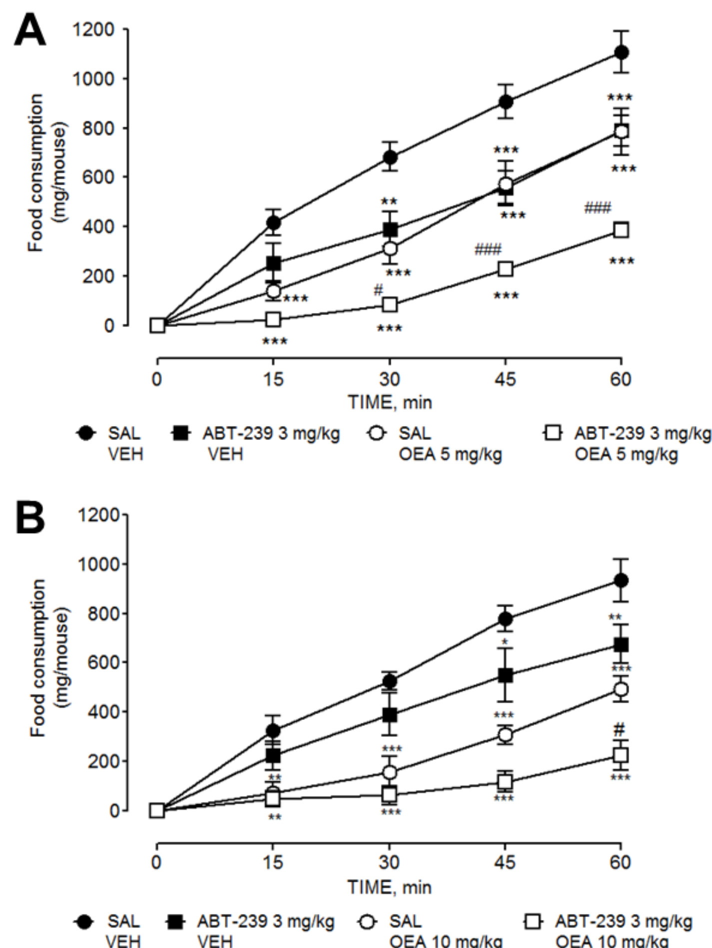

Figure III.4. Increased brain histamine boosts OEA-induced suppression of food intake. The graphs show the time course of OEA and ABT-239 effects on cumulative food intake in 12h-fasted CD1 mice. Different dose combinations of compounds and vehicles were used: (A) 5 mg/Kg OEA, 3 mg/Kg ABT239; (B) 10 mg/Kg OEA, 3 mg/Kg ABT-239. Each point represents mean cumulative food consumption ± S.E.M of 7-9 mice. Experiments were performed during the light-on period. ***P<0.001, **P<0.01, *P<0.05 vs respective controls; ###P<0.001; ##P<0.01, vs OEA treated mice by Two-way ANOVA and Bonferroni test.

Effects on central histamine release

We hypothesized that OEA may exert its effects at least in part, by modulating histamine signalling in the brain. Using in vivo microdialysis we found that systemic administration of 10 mg/kg OEA increased histamine release from the prefrontal cortex by approximately 160%. Furthermore, OEA increased histamine release only in 12h fasted, but not in satiated mice (Figure III.5A). The increase in histamine was transient and reached a maximum within the time frame of OEA effect on feeding behaviour. The mean spontaneous histamine release was not significantly different between experimental groups (53.7 ± 18.8 fmol/30 min fasted mice, n=3; 61.9 ± 33 fmol/30 min, fed *ad libitum* mice; n=4). We then measured histamine release from the cortex of freeely moving mice that received i.p. injection of ABT-239 (3 mg/kg). As shown in Figure III.5B, ABT-239 increased significantly histamine release from the mouse prefrontal cortex by approximately 130%. The mean spontaneous release of histamine was 49 ± 12 fmol/30 min (n=4). In order to support the further decrease in food intake shown in Figure III.4, we measured histamine release from the prefrontal cortex following i.p. administrations of both OEA (5 mg/kg, a sub-maximal hypophagic dose) and ABT-239 (3 mg/kg). Two-way ANOVA followed by Bonferroni's post hoc test showed the significant main effect of time and treatment between groups ($F_{(treatment)1,61}$=6.12, $P<0.01$; $F_{(time)8,61}$=11.6, $P<0.0001$; $F_{(treatment \times time)8,61}$=1.37, $P=0.227$). As shown in Figure III.5B, treatment with both compounds further increased cortical histamine release. The mean spontaneous histamine release was 34.3 ± 14.1 fmol/30 min (n=5).

To ascertain that our α-FMH protocol caused a prolonged depletion of releasable histamine stores from the mouse brain, we also measured histamine release from the mouse cortex 24h after αFMH i.c.v. injections. Cortical basal histamine contents of αFMH-treated mice were below detection level and no further histamine release was elicited when mice received ABT-239 (Figure III.5B).

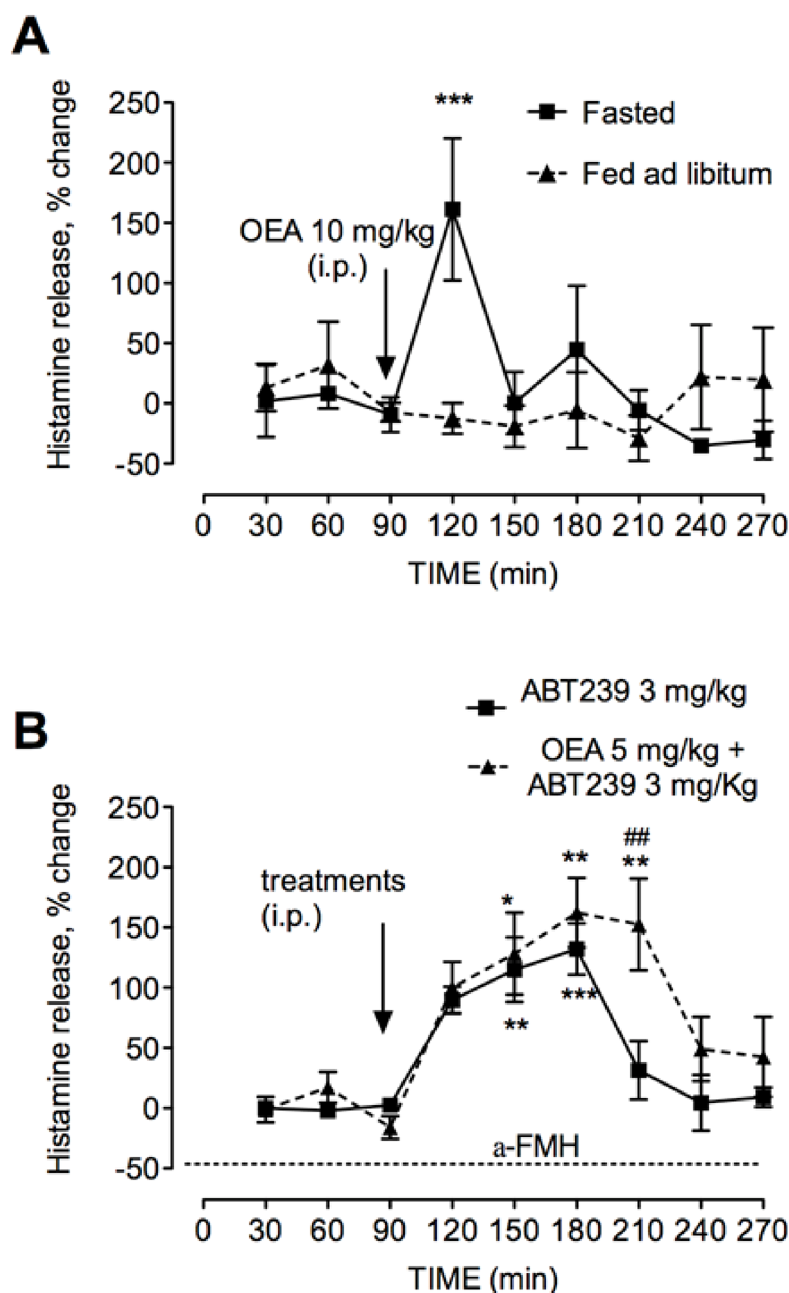

Figure III.5. Functional interactions between brain histamine and OEA. (A) Effect of OEA administration on histamine release from the prefrontal cortex of freely moving mice. Mean baseline histamine release was 53.7 ± 18.8 fmol/30 min in 12h fasted mice (n=3) and 61.9 ± 33 fmol/30 min in fed mice (n=4). (B) Effect of ABT-239 on cortical histamine release in freely moving mice. Mean spontaneous histamine release was 49 ± 12 fmol/30 min (n=4). OEA and ABT-239 were administered i.p. separately. Mean spontaneous histamine release was 34.3 ± 14.1 fmol/30 min (n=5). α-FMH was administered i.c.v (5 µg). Baseline histamine release in αFMH-treated mice was not detectable, nor during ABT-239 administration (n=4). Traced line (----) represents the methodological quantification limit. Experiments were carried out between 9:00h and 16:00h. ***P<0.001, **P<0.01, *P<0.05, vs baseline values; ##P<0.01 vs ABT-239 by two-way Anova and Bonferroni's test.

Induction of c-fos expression by OEA in WT and HDC-KO mice.

In the attempt to clarify how brain histamine deficit may prevent the satiety effect of OEA, we measured c-Fos protein expression in the brain of fasted HDC-KO and WT mice following OEA administrations (10 mg/kg, i.p.). The preferential sites of histamine-mediated suppression of food intake in the mammalian brain are the VMH and the PVN, as H1R ligands within these regions affect both meal size and duration. We found that OEA did not change c-Fos expression in the VMH ($F_{3,18}$ = 0.081, n.s.) of either WT or HDC-KO mice (Figure III.6C). As expected from previous results obtained in rats (Gaetani *et al.*, 2010), systemic OEA increased significantly c-Fos expression in the PVN of WT mice (Figure III.6D; $F_{3,18}$ = 4.817, $P<0.05$). However, no differences in the expression of c-Fos were found in the PVN of HDC-KO mice treated with vehicle or OEA. OEA activates prevalently oxytocin neurons in the PVN (Gaetani *et al.*, 2010). We found that the percentage of oxytocin neurons expressing c-Fos immunofluorescence was significantly lower in the PVN of OEA-treated HDC-KO mice compared to OEA-treated WT mice (Figure III.7; $P<0.01$) despite HDC-KO mice expressing higher levels of oxytocin-positive neurons (WT, 166 ± 17/mm^2 vs HDC-KO, 235 ± 14/mm^2; $P<0.05$, unpaired t-test). We also evaluated c-Fos expression in limbic brain structures related to feeding behaviour that receive histaminergic innervation (Airaksinen & Panula, 1988). Interestingly, in the nucleus accumbens that receives TMN input regulating exploratory behavior (Orofino *et al.*, 1999) indispensable during food provisioning, OEA administration decreased c-Fos expression in WT mice (Figure III.6B; $F_{3,16}$ = 5.550, $P<0.05$). In HDC-KO mice OEA decreased c-Fos expression to the same extent as in WT mice. In the infralimbic cortex, that presumably implements behavioural arousal during appetitive behaviour (Valdés *et al.*, 2006) and provides the TMN with an essential input for the appetitive function of histaminergic neurons no differences were found in c-Fos expression in either WT or HDC-KO mice receiving OEA (Figure III.6A; $F_{3,14}$ = 0.4947, n.s.).

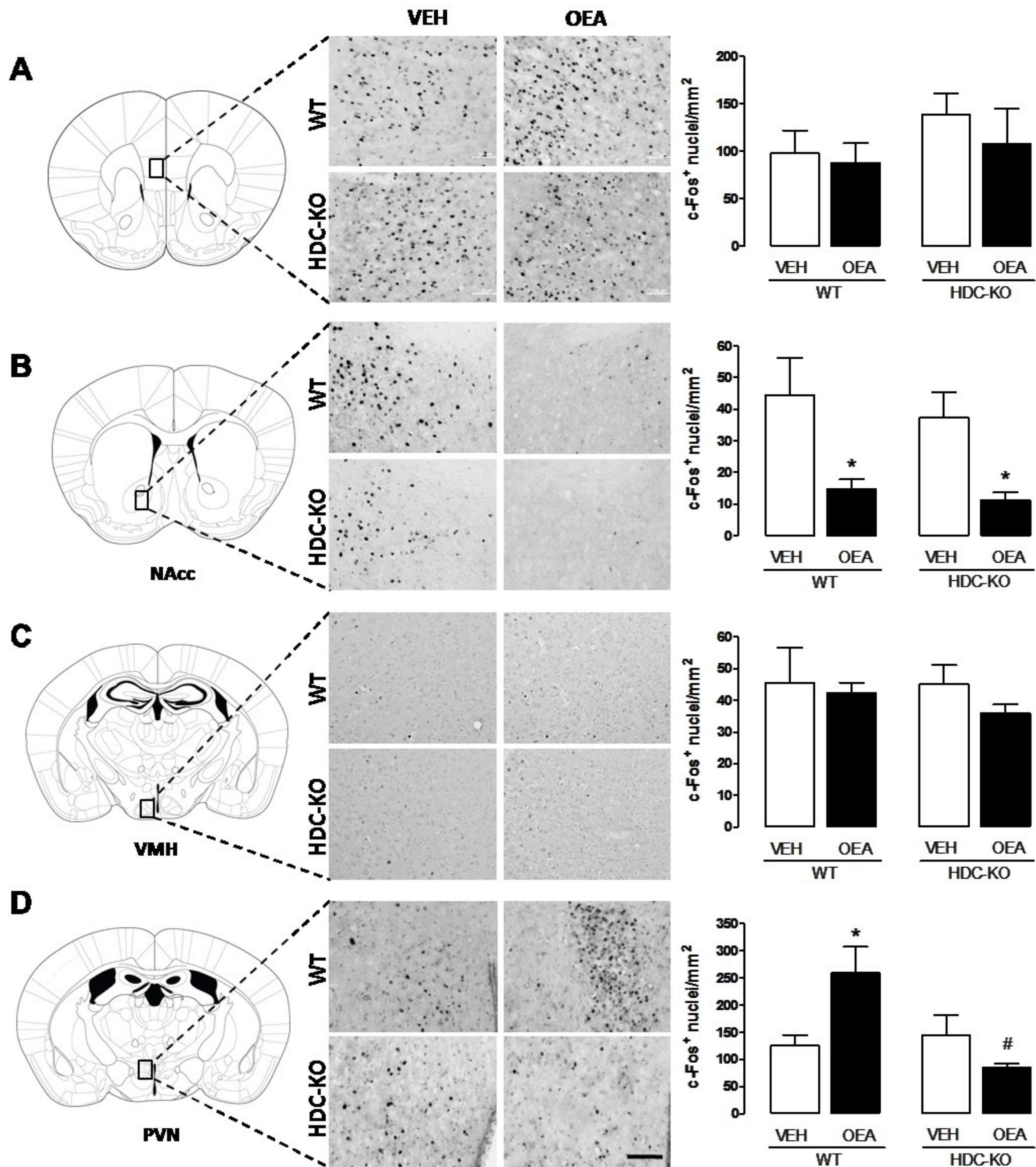

Figure III.6: OEA-induced c-Fos expression in the infralimbic and hypothalamic nuclei. Mouse brain coronal sections show the effect of vehicle or OEA (10 mg/Kg) on c-Fos protein expression in WT mice and HDC-KO mice in (A) infralimbic cortex – IL Cx (B) nucleus accumbens – Nacc, (C) ventromedial hypothalamus – VMH and (D) paraventricular nucleus – PVN. Calibration bar, 100 µm. Quantitative data are expressed in the graph bars; *P<0.05 vs respective controls; #P<0.05 vs WT/OEA by one-way ANOVA and Newman Keuls' test

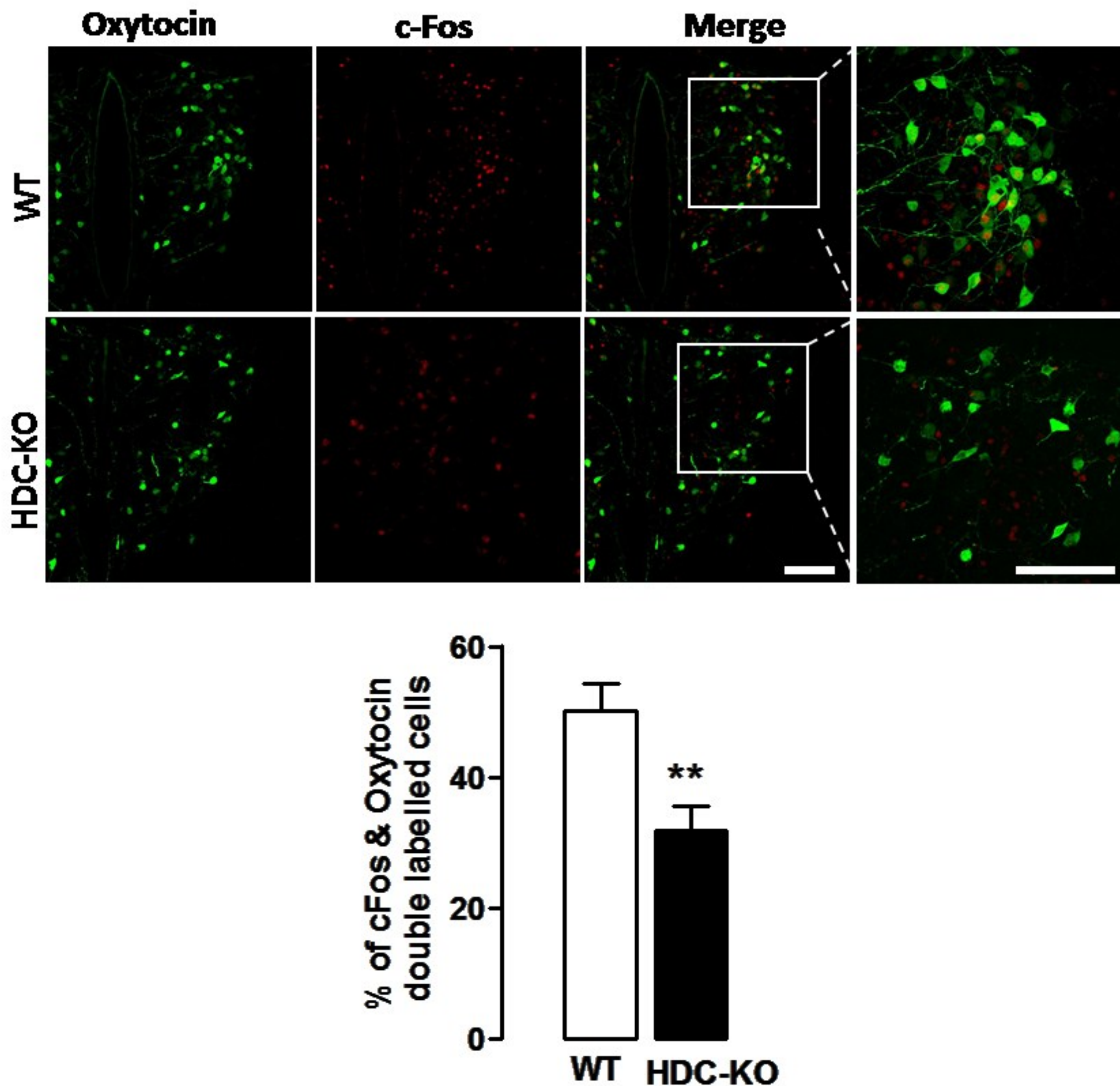

Figure III.7: OEA-induced c-Fos expression in PVN oxytocin neuron is blunted in HDC-KO mice. Immunohistochemical detection of oxytocin (left panels) and c-Fos (middle panels) in the PVN neurons of WT and HDC-KO mice treated with OEA. Mice were fasted for 12 h and sacrificed 120 min after OEA administration. Calibration bars, 50 µm. The bar graph shows the percentage of oxytocin immunopositive neurons that express c-Fos in OEA treated WT and HDC-KO mice; **P<0.01 by unpaired T test. N=3-5 mice.

DISCUSSION

Satiety-related signals are integrated at the cellular and system level to give reliable and appropriate behavioral responses by recruiting specific neuronal populations. OEA is synthesized in several peripheral tissues and the CNS (Izzo *et al.*, 2010) in mammals it has been described as a mediator of numerous metabolic processes (Thabuis *et al.*, 2008). OEA secreted by enterocytes serves as a fat-

sensing molecule that signals to satiety centers in the brain by engaging vagus nerve sensory fibers (Rodriguez de Fonseca *et al.*, 2001). OEA suppresses feeding by indirectly activating central oxytocin transmission in the PVN and SON (Gaetani *et al.*, 2010; Romano *et al.*, 2013a), and induces changes in hypothalamic monoaminergic turnover (Serrano *et al.*, 2011). However, it is not known if other neurotransmitter systems integrate the peripheral signalling of OEA with effector hypothalamic nuclei. Brain histamine affects feeding behaviour in a complex and not fully understood fashion (Figure H). It is fundamental for appetitive and aversive responses during motivated behaviour (Torrealba *et al.*, 2012), and blockade of H1R in the hypothalamus is responsible for the weight gain and metabolic dysregulation associated with the clinical use of atypical antipsychotics (Deng *et al.*, 2010). Several peptides and hormones such as corticotropin-, thyrotropin-releasing hormones and nefastin-1 function as satiety modulators and their hypophagic effects require the integrity of the brain histamine system (Gotoh *et al.*, 2005; 2007; Parmentier *et al.*, 2009; Gotoh *et al.*, 2013). So far, there was no information regarding brain histamine taking part in the anorexic effects of a modulator impermeant to the BBB such as OEA. Here, we show that chronic or acute histamine deficiency reduce significantly OEA-induced hypophagia, supporting our initial hypothesis that OEA requires the integrity of the histamine system to fully exert its hypophagic effect.

To understand which brain regions require histamine signaling for OEA-induced hypophagia, we used c-Fos expression as marker of functional activity. We found that lack of central histamine dampens OEA-induced increase of c-Fos expression in oxytocin PVN neurons of HDC-KO mice. The PVN integrates central and peripheral satiety signals, and H1R activation within the PVN has been implicated in the neuronal regulation of appetite (Figure III.8) as reported by decreased food intake following brain infusion of an H1R agonist, and increased c-Fos like immunoreactivity within the PVN (Masaki *et al.*, 2004). As pharmacological blockade of oxytocin receptors in the brain prevents OEA anorexiant effects (Gaetani *et al.*, 2010), we believe that histamine signalling on oxytocic PVN neurons is necessary for OEA to fully exert its hypophagic effect. In this regard, intranuclear and systemic release of oxytocin in response to suckling is controlled by H1R and H2R within the PVN (Bealer & Crowley, 2001; Bealer *et al.*, 2010). It was recently shown that noradrenergic NTS-PVN projections are

involved in the activation of the hypothalamic oxytocin system (Romano *et al.*, 2013b). In the TMN, α2 adrenoreceptors inhibit GABAergic transmission onto TMN neurons (Nakamura *et al.*, 2013). It is conceivable that NTS adrenergic fibres projecting to the TMN disinhibit TMN neurons that in turn facilitate oxytocin release from PVN to mediate OEA's pro-satiety effect.

Although the VMH appears also to be implicated in the histaminergic regulation of appetite (Masaki & Yoshimatsu, 2010; Clapp & Luckman, 2012), OEA did not induce c-Fos expression in this hypothalamic nucleus in WT mice, nor in HDC-KO mice. Interestingly, we found that OEA decreased neuronal activity within the NAcc, as suggested by the low expression of c-Fos in both HDC-KO and WT mice. In this context we did not investigate this aspect further, but it would be interesting to know if OEA, as a satiety factor, offsets brain centres that control the hedonic aspect of feeding (Berridge, 2009). To our knowledge the only other brain region where peripheral administration of OEA lowers neuronal activity is the lateral hypothalamus (Soria-Gómez *et al.*, 2010) where orexin neurons regulate feeding and sleep duration (Lin *et al.*, 2011). Brain histamine signalling in the PVN, therefore, seems to be involved in the acute effects of OEA on food consumption. In this case, we would expect that OEA could increase brain histamine release. Although technical limitations do not allow performing *in vivo* microdialysis experiments in the mouse PVN, we found in support of our hypothesis, that OEA increased cortical histamine with a time course compatible with its effects on feeding behavior.

We further tested our hypothesis of brain histamine and OEA signaling functional interactions, by measuring food consumption after co-administration of OEA and ABT-239. Previous reports indicate that H3R antagonists decrease food intake in several mammalian species (reviewed in (Hancock & Brune, 2005), and here we report for the first time such an effect for ABT-239 as well. Furthermore, ABT-239 increased cortical histamine, and consistent with feeding behavior we observed a further increase in histamine release following ABT-239 and OEA co-administration. Our results do not conclusively prove if the two systems act in a synergistic or additive fashion to control feeding behavior. We think that both components are at play. Presumably, the hypophagic effects of OEA and ABT-239 converge in the PVN, where increased histamine release activates H1R that in turn stimulate oxytocin release (Figure H). A crucial experiment would be to measure

histamine release using different dose combinations of OEA and ABT-239 from the PVN, which is unfeasible in the mouse. In addition, histaminergic neurons may also induce hypophagia by targeting brain regions not affected by OEA, such as the VMH (Ookuma *et al.*, 1993); also, H3R antagonists regulate the release of several neurotransmitters other than histamine (Passani & Blandina, 2011), which may contribute to the hypophagic effects of these compounds independently of OEA.

Brain histamine seems to have different roles during food anticipatory responses and food consumption (Ishizuka *et al.*, 2006; Torrealba *et al.*, 2012). Such a complex orchestration may be served by different histamine neuronal subpopulations that are recruited at different times during the unfolding of a specific behaviour (Blandina *et al.*, 2012). Histamine neurons send broad projections within the CNS that are organized in functionally distinct circuits impinging on different brain regions (Giannoni *et al.*, 2009; Giannoni *et al.*, 2010). This implies independent functions of subsets of histamine neurons according to their terminal projections and their selective participation in different aspects of behavioral responses. It is interesting that OEA elicits histamine release from the cortex of hungry mice, but it is ineffective in satiated animals. This is the first report that endogenous molecules affect histamine release differentially depending on the homeostatic state of the animal. Whether histamine modulation in the cortex is relevant for satiety, to curb the incentive to eat or else, is not known and certainly deserves further study.

We believe that understanding the role of the histaminergic system in driving or modulating feeding behaviour is of therapeutic relevance, as atypical antipsychotics, for instance, are thought to cause obesity by targeting histamine H1R (He *et al.*, 2013), and the orexigenic potencies of atypical antipsychotic drugs parallel their affinities for histamine H1R (Kroeze *et al.*, 2003; Coccurello & Moles, 2010). Our study may contribute to the development of more effective pharmacoterapies to treat obesity and to ameliorate the profile and side effects of centrally acting drugs.

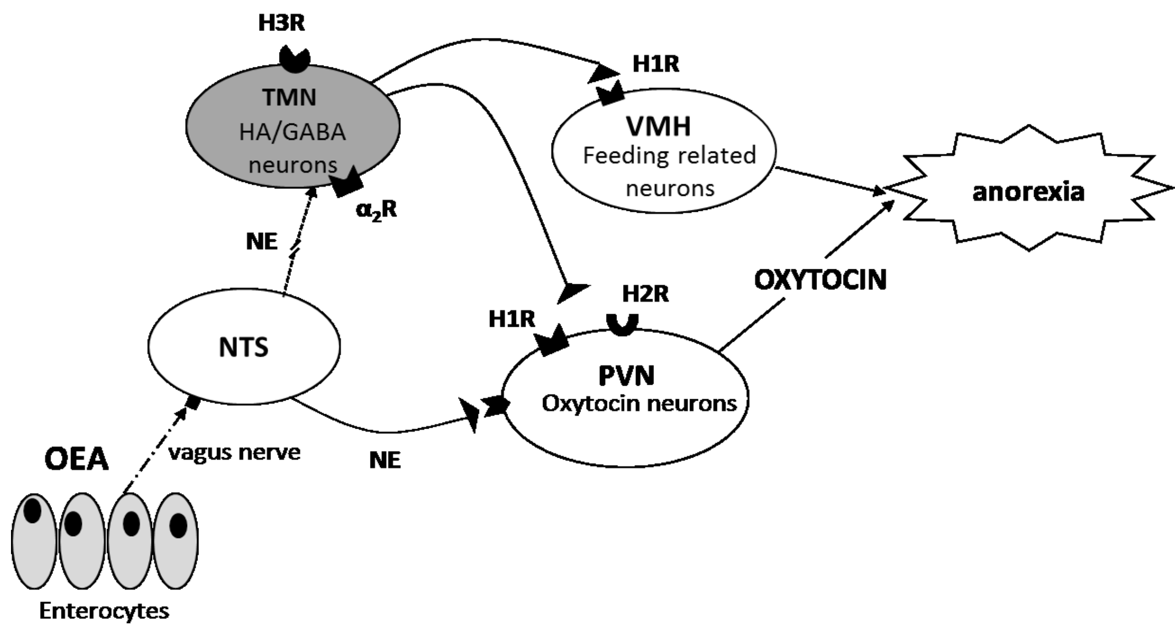

Figure III.8. Schematic drawing illustrating the putative interactions between OEA and the central histaminergic system. Histamine neurons are localized exclusively in the TMN of the posterior hypothalamus. Putative driver and modulatory inputs to the TMN are designated according to Torrealba et al (2012). The broken line designates presumed excitatory input from the nucleus of the solitary tract (NTS) to the TMN. According to our hypothesis, OEA induces anorexia indirectly stimulating histamine neurons in the TMN that project to the PVN. Activation of H_1 and H_2 receptors on feeding-related neurones in the PVN stimulates oxytocin release. Histamine mediates suppression of food intake also independently of OEA activating neurons in the VMH (Masaki & Yoshimatsu, 2006).

CONCLUDING REMARKS

Neuronal histamine is produced by neurons exclusively located in the tuberomamillary nucleus (TMN). These cells send widespread projections to almost all brain areas (haas 2008). This distribution pattern led to consider initially the histaminergic system as a homogeneous cell group regulating brain activity through diffuse projections rather than processing specific functions (Wada *et al.*, 1991). These days it is known that several brain functions are modulated by neuronal histamine: sleep-wake cycle, thermoregulation, feeding behaviour, arousal, emotionality and memory and cognition (Panula & Nuutinen, 2013) and there is growing evidence demonstrating that the histaminergic neurons are organized into distinct functional circuits that responds differentially to several stimuli (Blandina *et al.*, 2012). In the present work we provide further support to this hypothesis demonstrating that perfusion with the H3R antagonist-inverse agonist ABT-239 into the rat TMN increased histamine release and c-Fos expression in the prefrontal cortex and nucleus basalis magnocellularis, but not in the nucleus accumbens or dorsal striatum. Further research is required to understand the anatomical and physiological features that render the histaminergic neurons a heterogeneous population.

Based on the observation that ABT-239 induces activation of brain regions where histaminergic system modulates acetylcholine release resulting in changes in animal performance in learning tasks we decided to study the behavioural and neurochemical effects induced by ABT-239 and donepezil, an acetylcholinesterase inhibitor, in normal and histamine-deprived mice. Both compounds showed promnesic effects in normal mice mediated by the activation of the PI3/AKT/GSK-3β pathway. Histamine-deficiency impaired the procognitive effects and cortical and hippocampal GSK-3β phosphorylation elicited by both drugs, indicating that the integrity of the central histaminergic system is required for these effects.

Finally we observed an important interaction between a lipid-derived satiety factor, oleoylethanolamide (OEA), and the brain histaminergic system in the control of food intake. We found that histamine deprivation attenuated OEA hypophagic effect whereas increase in central histamine availability induced by ABT-239 potentiated it. Moreover, we observed that OEA induced c-Fos expression in the paraventricular nucleus of wild type but not in HDC-KO mice, thus we suggest that the hypophagic effects of histamine and OEA converge on oxytocic neurons in the hypothalamic paraventricular nucleus.

Our results indicate an interesting pharmacological profile for ABT-239 that could be extended to other H3R antagonist inverse/agonist: its precognitive effects associated with increase in GSK-3β phosphorylation suggest its potential utility in Alzheimer's disease therapy. This molecule could be also useful for obesity therapy since we demonstrated its hypophagic effects. Finally, an interesting therapeutic application of this compound could be in association with atypical antipsychotics, to reduce side effects induced by these drugs, like weight gain and to ameliorate cognitive deficits observed in schizophrenia patients. Moreover, the lack of effects observed in the striatum and nucleus accumbens following ABT-239 perfusion in the TMN suggests low potential for actions in the reward systems or extrapiramidal side effects. Unfortunately, prolongation of QT interval observed in monkeys after oral treatment with ABT-239 (although at doses expected to produce plasmatic concentratios 30-fold higher than the plasmatic effective concentration) precluded future clinical research for this compound (Hancock, 2006). Nonetheless, results from phase I and II clinical trials revealed that other H3R antagonists such as pitolisant, ABT-288, PF-03654746, MK-0249 were generally safe and well tolerated (Schwartz, 2011b; Zoethout *et al.*, 2012; Barchuk *et al.*, 2013; Othman *et al.*, 2013a; Othman *et al.*, 2013b). Therefore our observations remark the actractiviness of brain H3R as a target for the development of potentially innovative drugs for the treatment of cognitive impairments and/or eating disorders with better safety profile than the currently available compounds.

In conclusion, the histaminergic system continues to raise interest in the scientific community in light of its role in enabling the organism to cope with challenges and novelty, and in the physiology and patophysiology of cognitive and eating disorders.

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