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THE BRAIN HISTAMINERGIC SYSTEM AS A MODULATOR OF AVERSIVE MEMORY AND FEEDING BEHAVIOUR

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…Vola libera e felice,
al di là dei compleanni,
in un tempo senza fine, nel per sempre.
Di tanto in tanto noi ci incontreremo
-quando ci piacerà-
nel bel mezzo dell'unica festa
che non può finere mai…

"Nessun luogo è lontano", Richard Bach

Il tuo primo insegnamento, il mio primo libro,
Il tuo amore

A mia mamma

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INTRODUCTION

THE HISTAMINERGIC SYSTEM

History and breakthroughs in histamine research

2-(1H-imidazol-4-yl)ethanamine or histamine (Etymology: Gk, histos, tissue; L, amine, ammonia) is an endogenous amine occurring in several tissues (Haas *et al*, 2008) that has a variety of biological activity both in periphery and in the central nervous system. The first insight of the existence and the biological activity of this substance was furnished by Sir Henry Dale and his collaborators more then one century ago: after isolating histamine from the mould of ergot, they carried out experiments clarifying its biological actions, such as a stimulatory effect on the gut and the respiratory tract, the induction of cardiac contractility and the induction of a "shock-like syndrome", when injected in animals (Dale and Laidlaw, 1910, 1919). In 1920 the stimulant effect on gastric acid secretion on dogs was studied (Popielski, 1920) but it was Lewis that described the classic "triple response" to histamine, that consists of a red spot caused by vasodilatation, due to an increased permeability, and flare because of an axon reflex (Lewis, 1924). However it was only in 1972 that Best and colleagues could isolate histamine in samples of lungs and liver, indicating this amine as an endogenous constituent of the body (Best *et al*, 1927).

Histamine in the central nervous system was described by Kwiatkowski (1943) and White (1959), mostly in grey matter, by demonstrating its synthesis and catabolism in the brain (Kwiatkowski, 1943)Observing the classical sedative side effect of antihistamines (Bovet and Staub, 1937; White, 1959) later induced scientists to study histamine as a "waking substance" (Monnier *et al*, 1967). Soon, other studies showed more details about histamine's synthesis and turnover in the brain (Pollard *et al*, 1974; Schwartz *et al*, 1970; Taylor and Snyder, 1971, 1972). However, while in the '60s experimental data lead to a better understanding of anatomical and functional features of the other biogenic amines in the brain, a strong in-depth analysis of the central histaminergic system was missing because of unsuitability of the techniques. Therefore brain histamine was neglected for quite some time. Studies on the effect of histamine or its antagonists on nerve cells in different regions of the central nervous system or on behaviour after intracerebrovascular (i.c.v.) infusion or in distinct brain regions proposed histamine as a neurotransmitter but lack of evidences made its recognition slow (Green, 1970). The breakthrough was in 1984 when the source of central histamine was described with immunohistochemistry techniques: the tuberomamillary nucleus in the posterior hypothalamus was found as the sole origin of the widely distributed histaminergic projections in the brain (Panula *et al*, 1984; Watanabe *et al*, 1984).

The statement that histamine has an active role in anaphylaxis stimulated the scientific community to look for antihistamines: Parrot and Bovet, in 1937, discovered the first substance, the piperoxan, capable of blocking the deleterious anaphylaxis effects of histamine in the guinea-

pig ileum (Bovet *et al*, 1937; Parsons and Ganellin, 2006), even though this molecule was later proved to be toxic for clinical use. In 1957 Bovet was awarded the Nobel Price for Medicine for his work on antihistamines and curare and he stated in its lecture: "considering the number of features that histamine, acetylcholine, and epinephrine have in common, we looked for antagonism comparable to that exhibited by sympatholytic compounds toward epinephrine and by parasympatholytic compounds towards acetylcholine". In 1942 the first antihistamine for human use, Antergan™ (phenbenzamine) was registered later replaced by Neoantergan™ (mepyramine, pyrilamine), still used topically in skin inflammatory diseases. After 1945, antihistamines, later called H1 receptor (H1R) antagonists, became very common drugs for treatment of various allergic disorders (Parsons *et al*, 2006).

With the advent of the use of antihistamines it became apparent that not all the described actions of histamine were antagonized by these molecules and this evidence, together with other scientific studies (Ashford *et al*, 1949; Folkow and Uvnas, 1948), induced Folkow to suggest for the first time that "there are two types of receptors sensitive to histamine" and that only one of these was responsive to the substance that he and his collaborators used. Crucial for the study of histaminergic receptors was the work of Schild (Ash and Schild, 1966).

In 1966, histamine receptors were first differentiated into H1- and H2 receptors (H2R) (Ash and Schild, 1966). In the late 1970s and 1980s, the H2Rs antagonists revolutionised the treatment of peptic ulcer and other gastric acid-related disorders. In 1999, a third histamine receptor subtype was discovered and termed H3 (H3R) (Lovenberg *et al*, 1999): the H3Rs antagonists, although available since 1987, have been slower to find a therapeutic role, now these substances provide possible alternatives in the treatment of obesity and a variety of central nervous system disorders like memory, learning deficits and epilepsy (Parsons *et al*, 2006): in 2013, pitolisant, an H3-R antagonist/inverse agonist, was authorized by EMEA for the treatment of narcolepsy . Finally, in 2000, the fourth histamine receptor was cloned, H4 (Oda *et al*, 2000): this discovery had the potential to provide drugs acting on the immunological system with possible applications in asthma and inflammatory diseases (Parsons *et al*, 2006).

Metabolism of histamine in the brain: synthesis, transport and inactivation of its action

The presence oh histamine in the brain can be found in two major pools: neurons and mast cells (Garbarg *et al*, 1976). The amount of mast cells in the central nervous system is poor compared to other tissues but, since up to 50% of histamine contents in the brain are from brain mast cells, mediators from brain mast cells may significantly influence sleep and other behaviours (Chikahisa *et al*, 2013). Differences in quantity of these cells are found depending on species, sex and on the physiological state (Dropp, 1979; Theoharides, 1990). A significant number could be found in the thalamus and hypophysis but elsewhere histamine actions are presumably mediated via its release from neurons (Hough, 1988).

Like in peripheral tissues, histamine in the central nervous system is synthetized from L-histidine by histidine decarboxylase (HDC) enzyme. The regulation of the HDC gene in the brain is under study and still not well understood; however, peptides like gastrin and pituitary adenylate cyclase-activating polypeptide (PACAP) (McLaughlin *et al*, 2004), steroids and other factors change the activity of HDC gene promoter (Ai *et al*, 2004; Fleming *et al*, 2004; Höcker *et al*, 1998; Nakagawa *et al*, 1997; Prinz *et al*, 2003; Zhang *et al*, 1996). The rate of histamine synthesis, depends on the bioavailability of the precursor L-histidine: this amino acid is brought into the cerebrospinal fluid and neurons via L-amino acids transporters (Haas *et al*, 2008).

In the central nervous system histamine is stored in cell somata and in axon varicosities (Diewald *et al*, 1997; Hayashi *et al*, 1984; Kuhar *et al*, 1971; Martres *et al*, 1975; Wouterlood and Gaykema, 1988a): from here histamine is transported in vesicles by vesicular monoamine transporter VMAT-2 (Ericson *et al*, 1987; Merickel and Edwards, 1995; Weihe and Eiden, 2000) and released after the arrival of the action potential (Haas *et al*, 2008). Although histamine concentration in the brain is lower compared to the other amines, its turnover is faster and changes depending on the functional state (Dismukes and Snyder, 1974; Pollard *et al*, 1993): histamine levels measured by in vivo microdialysis are higher during waking periods, according with the firing of histamine neurons (Mochizuki *et al*, 1992). The synthesis and the release of this biogenic amine are controlled by H3 autoreceptors located in cell somata and axonal varicosities via negative feedback (Arrang *et al*, 1983; Prast *et al*, 1992; Schwartz *et al*, 1991). Histamine release is also regulated by other neurotransmitters that modulate histamine firing rate or release from varicosities in a inhibitory fashion, via M1 muscarinic, α2 andrenergic, 5-HT1A, opioid k-, galanin and peptidergic receptors (Arrang *et al*, 1991; Gulat-Marnay *et al*, 1989a, b, 1990; Itoh *et al*, 1998; Itoh *et al*, 1988; Oishi *et al*, 1992). M-opioid receptors seem to have a facilitatory action in controlling histamine release

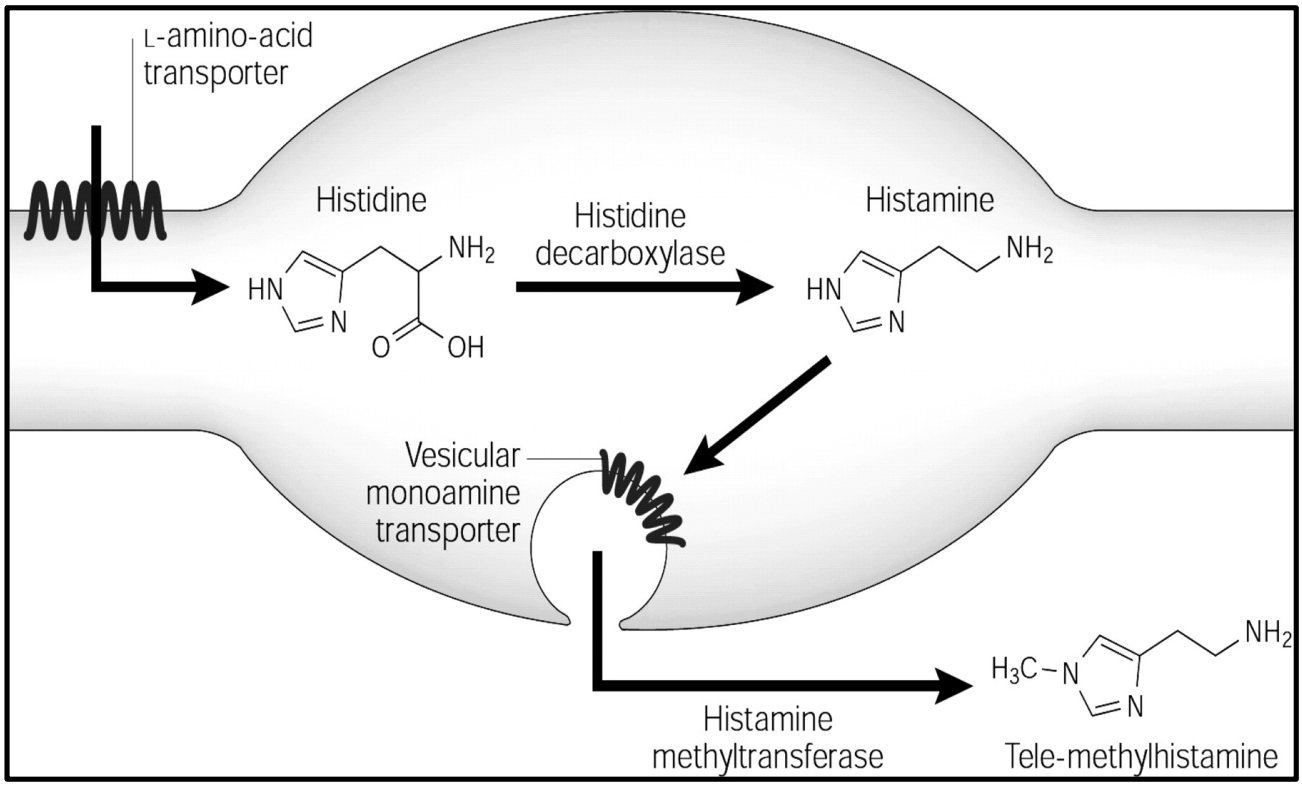

Figure 1. Histamine synthesis and metabolism. Histidine is taken up in a varicosity and decarboxylated; histamine is transported into a vesicle, released, and methylated (Haas et al., 2008)

(Itoh *et al*, 1988). Studies show that NO, a gaseous messenger molecule, can inhibit histamine release in the hypothalamus in vivo (Prast *et al*, 1992).

The catabolism of central histamine in the extracellular space is mediated by N-methyltransferase (Barnes and Hough, 2002; Bowsher *et al*, 1983; Matuszewska and Borchardt, 1983) and this methylation requires S-adenosyl-methionine, as a methyl donor (Green *et al*, 1987; Prell and Green, 1986; Schwartz *et al*, 1991) as a matter of fact blockers of HNMT diminish tele-methylhistamine and enhance histamine levels in the brain (Duch *et al*, 1978). Histamine does not readily pass the blood-brain barrier (Schwartz *et al*, 1991) and HNMT was also found in the walls of blood vessel where blood-synthesized histamine and mast cells histamine is deactivated (Nishibori *et al*, 2000). Moreover, a shuttle transport system from the brain to vasculature can drain neuronal histamine in excess. In the central nervous system, tele-methylhistamine undergoes oxidative deamination via monoamine oxidase (MAO-B) to t-methyl-imidazolacetic acid (Lin *et al*, 1993; Prell *et al*, 1988; Schwartz *et al*, 1991). In peripheral tissues and in invertebrates the main enzyme that catabolizes histamine, as already seen, is diamine oxidase (DAO), while in the brain its activity is low under basal conditions but when HNMT is inhibited it may be a collateral and salvage pathway for the production of imidazolacetic acid, a $GABA_A$ receptor agonist (Hösli and Haas, 1971; Prell *et al*, 1997).

Histaminergic receptors

Histamine elicits its actions via 4 metobotropic receptors: H1-, H2-, H3- and H4Rs have been cloned so far. H1-, H2- and H3Rs are expressed abundantly in the brain, H4Rs are expressed mainly in peripheral tissues and the presence of these receptors in the brain still remains controversial (Schneider *et al*, 2015). All four histaminergic receptors belong to the rhodopsin-like family of G-protein-coupled receptors (Hayashi *et al*, 1984; Hill *et al*, 1997; Leurs *et al*, 2005) they consist of seven large trans-membrane domains with prototypic regions that are needed for antagonist binding specificity and receptors activation (Bakker *et al*, 2007; Jongejan *et al*, 2005; Leurs *et al*, 2002; Lim *et al*, 2005), G protein coupling and constitutive activity (Bakker *et al*, 2004; Gbahou *et al*, 2003), but also for covalent modifications, homo- and eterodimerization, anchoring and membrane trafficking, and sensitization and desensitization (Kuramasu *et al*, 2006).

H1 receptors

The H1R is encoded by a gene located on human chromosome 3 (Jongejan *et al*, 2005). Its transduction mechanism is (Leurs et al., 1994) a $G_{alpha\ q/11}$ protein-coupled receptors (Bakker *et al*, 2004; Brown *et al*, 2002; Eriksson *et al*, 2001; Selbach *et al*, 1997): activation of phospholipase C (PLC) that induces inositol triphosphate (IP3)-dependent Ca^{2+} release from intracellular storage and diacyglycerol (DAG)-sensitive activation of protein kinase C (PKC), which helps the entry of Ca^{2+} through voltage-dependent calcium channels (VDCC) (Brown *et al*, 2002; Sergeeva *et al*, 2003a) and the stimulation of NCX Na+/Ca2+ exchanger (Eriksson *et al*, 2001; Sergeeva *et al*, 2003b).

Other effects of H1 histaminergic receptor activation comprehend the production of arachidonic acid (AA), nitric oxide (NO) and cGMP (Leurs *et al*, 1994; Prast *et al*, 1992; Richelson, 1978; Snider *et al*, 1984), via pertussis toxin-sensitive G_i/G_o protein-mediated activation of phospholipase A2 (PLA2). It is important to note that the H1R also activates AMP-kinase, fundamental in controlling energy metabolism (Kim *et al*, 2007), and nuclear factor kappaB (NF-kB) (Bakker *et al*, 2001), that controls genomic imprints and readout.

H1R in the brain

H1Rs are distributed in the peripheral tissues and central nervous system differently depending on species (Chang *et al*, 1979). High presence oh these receptors were found in brain regions involved in neuroendocrine, behavioural and nutritional state control, such as hypothalamus, aminergic and cholinergic brainstem nuclei, thalamus and cortex. In human, the highest concentration of

H1Rs, studied by using 3Hmepyramine binding, was found in cerebral cortex and infralimbic structures (Martines-Maldonado *et al*, 1975).

Via H1-Rs histamine excites neurons in most brain regions, including brainstem (Bárbara *et al*, 2002; Korotkova *et al*, 2005; Lin *et al*, 1996), hypothalamus, thalamus (Zhou *et al*, 2006), amygdala, septum (Dai *et al*, 2007; Gorelova and Reiner, 1996), hippocampus (Canto-de-Souza *et al*, 2015; Manahan-Vaughan, 2000), olfactory bulb (Jahn *et al*, 1995) and cortex (Reiner and Kamondi, 1994).

The complex H1R signal includes bidirectional and synergistic effects (Bakker *et al*, 2004; Dai *et al*, 2006; Garbarg and Schwartz, 1988; Leurs *et al*, 1994), as an example, H1R could oppose or amplify H2R actions depending on timing and context of activation and may serve as a coincidence detector for a Gsalpha-/PKA-dependent signalling (Bakker *et al*, 2004; Baudry *et al*, 1975; Garbarg *et al*, 1988; Selbach *et al*, 1997).

It is noteworthy that H1R-KO mice show immunological, metabolic and behavioural abnormalities (Hirai *et al*, 2004; Huang *et al*, 2003; Masaki and Yoshimatsu, 2006; Parmentier *et al*, 2002).

All H1R antihistamines act as inverse agonists, stabilizing the receptor into the inactive state (Bakker *et al*, 2004; Jongejan *et al*, 2005; Leurs *et al*, 2002). Classic antihistamines have well known sedative properties (Bovet, 1950; D, 1950; Lin *et al*, 1996; Reiner *et al*, 1994); of note, many antidepressants and antipsychotic also bind H1Rs (Kim *et al*, 2007; Richelson, 1978).

H2 receptors

The gene for H2R in human is located on chromosome 5 and encodes for a 7-transmembrane G-coupled protein (Traiffort *et al*, 1995). The $G_s\alpha$ G-protein is associated with H2R and its activation leads to the stimulation of adenylyl cyclase with consequent augmentation of intracellular second messenger cAMP (Bakker *et al*, 2004; Baudry *et al*, 1975; Garbarg *et al*, 1988; Traiffort *et al*, 1992). One of the targets of cAMP is the cAMP-dependent PKA that can phosphorylate proteins in the cytosol, cell membrane or can translocate in the nucleus where it activates the transcription of CREB (Sheng *et al*, 1991). All of these are fundamental regulators of neuronal physiology and plasticity. cAMP can also directly interact with activated cation channels Ih (HCN2) (McCormick and Williamson, 1991; Pedarzani and Storm, 1995). Via the PKA-dependent phosphorylation, histamine can block Ca^{2+}-activated potassium conductance (Atzori *et al*, 2000). Independently from cAMP or the intracellular levels of calcium, H2Rs also inhibit PLA2 and the release of arachidonic acid, which could explain the opposing physiological responses of H1 and H2 receptors in many tissues (Traiffort *et al*, 1992).

H2 in the brain

Like H1, H2R is present in the central nervous system and spinal cord (Traiffort *et al*, 1992; Vizuete *et al*, 1997) with a widespread but more consistent distribution than H1Rs: in particular high densities are found in the basal ganglia and parts of the limbic system, such as the hippocampus, the amygdala and cortex, where they show a laminar distribution, but compared to the quantity of H1Rs, H2 are present in lower densities in septal areas and thalamic and hypothalamic nuclei. H1- and H2Rs show strong colocalizations in different areas, including pyramidal and granule cells in the hippocampal formation and in other aminergic cells (locus coeruleus, raphe nuclei, substantia nigra, ventral tegmental area), (Bakker *et al*, 2004; Baudry *et al*, 1975; Garbarg *et al*, 1988; McCormick and Williamson, 1989; Selbach *et al*, 1997). In several brain regions, the activation of H2Rs leads to a depression of firing (Haas, 1974; Haas and Wolf, 1977) but much about this depression remains unexplained.

Mice deficient in H2Rs present specific cognitive deficits along with an impaired LTP in the hippocampus (Dai *et al*, 2007), abnormalities in nociception (Mobarakeh *et al*, 2006; Mobarakeh *et al*, 2005), in gastric functions and immune system (Teuscher *et al*, 2004).

H2 antagonist drugs are widely used in clinic for the therapy of gastric diseases and antitumor activity has been reported (Lefranc *et al*, 2006). Some antidepressant molecules also show H2R antagonistic properties (Green and Maayani, 1977).

H3 receptors

The H3R has been described by Arrang and collaborators in 1983 as an autoreceptor that controls the release and the synthesis of histamine (Arrang *et al*, 1983). The gene encoding human H3R is located con chromosome 20: this gene, differently from H1Rs' and H2Rs', codifies for large number of receptor isoforms that have different distribution and pharmacology (Bakker *et al*, 2006; Drutel *et al*, 2001; Tardivel-Lacombe *et al*, 2000). The H3R is (Hill *et al*, 1997), a G-protein coupled receptor pertussis-toxin sensitive (Gi/o), similar to many presynaptic inhibitory receptors (Brown *et al*, 2001b). Interestingly this receptor presents a low homology to many other biogenic amine receptors (Lovenberg *et al*, 1999). The H3R is negatively coupled with a pertussis toxin-sensitive $G_{i/o}$ and inhibits Ca^{2+} channels and adenylyl cyclase (Moreno-Delgado *et al*, 2006). Cross-talking with other GPCRs, H3-Rs also engages Gq/11 protein signal and activate PLA2, Akt/GSK3 (Bongers *et al*, 2007) and, indirectly, MAP kinase pathway (Giovannini *et al*, 2003), playing an important role in axonal and synaptic plasticity and in different disorders of the CNS.

A peculiar property of the H3Rs is their high degree of constitutive activity in vivo (Gbahou *et al*, 2003; Morisset *et al*, 2000). The existence of different forms active states constitutively active H3

states define a new pharmacological entity known as protean agonists, with strongly functional or therapeutic implications (Gbahou *et al*, 2003; Leurs *et al*, 1994; Stark *et al*, 2001).

H3 in the brain

To localize H3Rs in rat brain 3H(R)α-methylhistamine was used (Pollard *et al*, 1993): these receptors are present in all regions and layers of the cerebral cortex. High density was also found in the nucleus accumbens, striatum, olfactory tubercles and in the substantia nigra, in the hypothalamus only moderate levels have been described. H3Rs are present on somata of histaminergic neurons in the TMN (Blandina *et al*, 2012).

Since the original demonstration that the activation of H3Rs inhibits histamine synthesis and release, (Arrang *et al*, 1983), it has been found that these receptors can inhibits the release of other neurotransmitters including glutamate (Brown and Reymann, 1996), GABA (Garcia *et al*, 1997), noradrenaline (Schlicker *et al*, 1989), dopamine (Schlicker *et al*, 1993) and various other peptides (Hill *et al*, 1997).

The EC50 of histamine for the H3R is at least 10 times lower than for H1 and H2. A value of 40 nM was described for histamine inhibition of its own release in rat (Arrang *et al*, 1983) or in human (Arrang *et al*, 1987), whereas histamine inhibits high-threshold calcium channels with an EC50 of 260 nM (Takeshita *et al*, 1998). This difference could be explained with the presence of different receptor subtypes (Clapham and Kilpatrick, 1992; Cumming and Gjedde, 1994; Leurs *et al*, 1996).

H3-KO mice present behavioural state dysfunctions, reduced locomotion (Toyota *et al*, 2002), a metabolic syndrome with hyperphagia, late-onset obesity, increased insulin and leptin levels (Tokita *et al*, 2006; Yoshimoto *et al*, 2006) and an increased severity of neuroinflammatory diseases (Teuscher *et al*, 2007).

Atypical neuroleptics, like clozapine, bind H3 receptors. With these peculiar pharmacological properties, H3R is an important target for drugs against different disorders of the CNS (Passani and Blandina, 2011).

H4 receptors

The fourth histamine receptor was described two decades ago (Raible *et al*, 1994) and was cloned, starting from H3R sequence, by different groups, independently (Liu *et al*, 2001; Morse *et al*, 2001; Nakamura *et al*, 2000; Nguyen *et al*, 2001; Oda *et al*, 2000; Zhu *et al*, 2001). It is especially expressed on immune cells and has entered clinical studies as a potential therapeutic target for pruritus (Gutzmer *et al*, 2011). This receptor is coupled with a $G\alpha_{i/o}$ PTX-sensitive protein whose activation inhibits levels of cAMP forskolin-inducted and that has a fundamental role in gene

modulation of cAMP-responsive elements (Liu *et al*, 2001; Oda *et al*, 2000; Zhu *et al*, 2001). H4R-mediated signalling pathways levels of intracellular Ca^{2+} (Hofstra *et al*, 2003).

Its expression on immune cells is a well documented (Schneider *et al*, 2015)but, surprisingly, little is known about the presence and the role of H4Rs in the CNS. Some groups found the expression of H4R mRNA in brain tissue (Liu *et al*, 2001; Strakhova *et al*, 2009; Zhu *et al*, 2001), while others obtained negative results (Morse *et al*, 2001; Nakamura *et al*, 2000; Nguyen *et al*, 2001; Oda *et al*, 2000). Protein expression of this receptor was suggested in the brain (Connelly *et al*, 2009; Lethbridge and Chazot, 2010) but these finding are still controversial (Beermann *et al*, 2012; Gutzmer *et al*, 2012).

Tuberomamillary nucleus

Generalities, anatomy and cellular morphology

The central histaminergic system is well preserved through phylogeny with comparable morphological and functional features: it is a system that activates different brain areas according to the environmental and metabolic state of the animal, such as feeding-related arousal and waking and attention in vertebrates (Ferrer *et al*, 1979; Niimi *et al*, 1997).

Studies using electrophysiological techniques hypothesized the existence and the location of tuberomamillary nucleus (TMN) (Dismukes *et al*, 1974; Garbarg *et al*, 1974; Haas, 1974; Haas *et al*, 1977; Vorobjev *et al*, 2003) but only immunohistochemistry, using anti-HDC antibody, was able to clearly visualize the histaminergic hypothalamic nucleus (Ericson *et al*, 1987; Köhler *et al*, 1986; Panula *et al*, 1984).

The TMN is localized anterior to the mamillary bodies and the chiasma opticum in the tuber cinereum. In rat central nervous system, Ericson and colleagues (Ericson *et al*, 1987) evidenced a ventral subdivision (TMV, ≈1500 neurons each side), a medial (TMM, ≈600 cells each side) and a spread area (≈200 scattered neurons). Inagaki and co-workers (Inagaki *et al*, 1991; Inagaki *et al*, 1990) then described a fifth part subdividing the TMV rostral and caudal and the TMM in dorsal and ventral (E1-E5). There are evidences for heterogeneity within histaminergic neurons: for instance it is possible to describe different responses to environmental stimuli and stress (Miklós and Kovács, 2003), endocannabinoids (Cenni *et al*, 2006), GABA and glycine (Sergeeva *et al*, 2002).

In mouse brain TMN is less compact and is characterized by smaller and fewer neurons compared to rat's ones (Parmentier *et al*, 2002).

The human histaminergic system is extensive with ≈64000 neurons. A complete and detailed analysis of histaminergic projections is not available yes but high densities have been found in the

cortex, in lamina I (Panula *et al*, 1990). In rodents, the dendrites of TMN neurons make connections with brain surface, whereas in human, in posterior hypothalamus, varicose axons accumulate in the area. Similarly to rat, in human brain it is possible to discern fours subunits in the TMN: a major ventral part, actually the tuberomamillary nucleus proper, a medial part that includes also the supramamillary nucleus, a caudal paramamillary area, and a minor lateral one (Airaksinen *et al*, 1991).

The morphological characteristics of histaminergic neurons somata are comparable between species and also similar to aminergic ones in the mesencephalon. They mostly possess big somata (25-30 µm), with two or three dendrites (Wouterlood *et al*, 1986) that meet with dendrites of other histaminergic neurons.

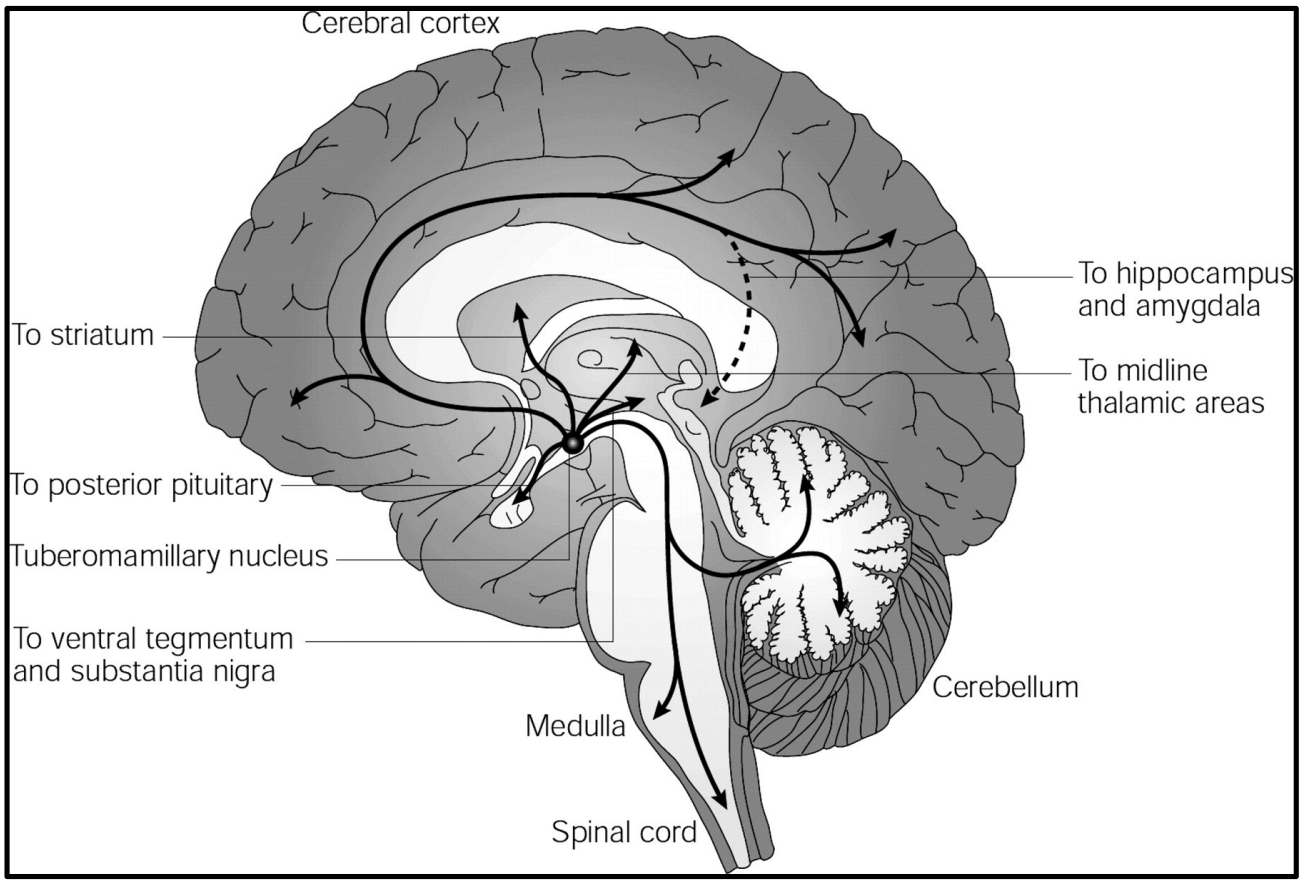

Figure 2. The histaminergic system in the human brain. The histaminergic fibers emanating from the tuberomamillary nucleus project to and arborize in the whole central nervous system (Haas et al., 2008).

Heterogeneity of histaminergic neurons in the TMN

Heterogeneity of histamine neurons in response to stress

Histamine release is a sensitive indicator of stress (Verdière *et al*, 1977; Westerink *et al*, 2002). Indeed, restraint and/or metabolic stress are among the most potent activators of histamine neurons. Different types of stress (Miklós *et al*, 2003), as well as hypercapnic loading (Haxhiu *et al*,

2001) activated only selected subgroups of histaminergic neurons. These findings rely on the measurement of *c-Fos* immunocytochemistry, a marker of cell activation, in histamine neurons identified with *in situ* hybridization of HDC mRNA. Under stress-free, basal conditions *c-Fos* expression was detected in a negligible number of histamine neurons (less than 1%) distributed uniformly in the TMN (Miklós *et al*, 2003). Following restraint, up to 36% of histamine neurons in E4 and E5 regions became *c-Fos* positive, but significantly fewer, less than 10%, were activated in E1, E2, or E3 regions (Miklós *et al*, 2003). Moreover, different types of stress, foot shock or insulin-induced hypoglycemia, activated E4 and E5 histamine neurons, but failed to induce any significant *c-Fos* activation in the histamine neurons of the three remaining clusters. Therefore, stress-sensitive histamine neurons were detected in the rostral (E4–E5) subgroups rather than in the caudal ones (E1–E3), clearly indicating that histaminergic neurons of distinct TMN clusters are recruited in a stressor- and subgroup-specific manner (Miklós *et al*, 2003).

In line with the concept of heterogeneity of these neurons, only a subset of histaminergic neurons (E2) responded to hypercapnic stress (CO_2 exposure) with an increase of *c-Fos* expression (Haxhiu *et al*, 2001; Johnson *et al*, 2005). Consistently, recordings in rat brain slices revealed that acidification within the physiological range 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). The functional significance of chemosensory traits in histaminergic neurons is not well known. However, it is expected that activation of histamine neurons by an increase in CO_2 and/or H^+ may affect central respiratory drive through activation of neurons in the nucleus tractus solitarius, a region displaying a dense network of histaminergic fibres (Airaksinen *et al*, 1989; Airaksinen and Panula, 1988).

Heterogeneity of histamine neurons in response to GABA and glycine

Heterogeneity within the histaminergic neuron population was shown also using a different approach. It is well established that the activity of histamine neurons is closely associated with the behavioural state: in behaving cats, rats, and mice, the firing is more variable during waking and absent upon drowsiness and during sleep. This is the most wake-selective firing pattern identified in the brain to date (Lin, 2000). Sleep-active, GABAergic neurons in the ventrolateral preoptic nucleus (VLPO) provide a major input to the TMN, and may play a key role in silencing the ascending histaminergic arousal system during sleep (Sherin *et al*, 1998). Interestingly, GABA release in the posterior hypothalamus increased during slow waves sleep (SWS), and microinjection of the GABAA-receptor (GABAA-R) agonist muscimol, into the same area increased

SWS time (Nitz and Siegel, 1996). Hence, GABA release in the posterior hypothalamus inhibits directly histaminergic cells firing rate (Yang and Hatton, 1997), thereby facilitating SWS, whereas GABAA-R inhibition increases significantly the release of histamine from the TMN, as measured with microdialysis (Cenni *et al*, 2006). Electrophysiological studies using whole-cell recording identified two subpopulations among histamine neurons isolated from the TMN according to their sensitivity to GABA (Sergeeva *et al*, 2002), with GABA concentrations for threshold and maximal responses ranging between 0.5 and 5 µM in one subpopulation, and between 50 and 500 µM in the other one (Sergeeva *et al*, 2002). GABA responses were completely blocked by gabazine, a selective antagonist of GABAA-R (Sergeeva *et al*, 2002). This receptor is a protein complex assembled from a family of 19 homologous subunit gene products that form mostly hetero-oligomeric pentamers. The major isoforms contain alpha, beta, and gamma subunits and show differential sensitivity to GABA, to modulators like steroids, to physiological regulation, and to disease processes (Olsen and Sieghart, 2009). Sergeeva and coworkers (Sergeeva *et al*, 2002), using whole-cell recording and single cell RT-PCR from isolated rat histamine neurones, characterized GABAA-R evoked currents and correlated them with the expression patterns of 12 GABAA-R subunits. They identified three different groups of histamine neurons on the basis of their gamma subunits expression. The occurrence of each gamma subunit was correlated with GABA EC50. The group expressing both gamma1 and gamma2 subunits displayed a high sensitivity to GABA, whereas the group expressing only the gamma2 subunit displayed a low sensitivity. Histaminergic neurons are also heterogeneous with respect to their sensitivity to glycine that correlates with their size. Indeed, the maximal glycine response (1 mM) in histaminergic cells with larger somata (25 µm) was about half of the maximal GABA response whereas in the cells with a smaller soma size (19.5 µm) the glycine response was absent or very small (Sergeeva *et al*, 2001).

Histamine neurons establish functionally distinct pathways according to their terminal projections

Blandina and co-workers (Blandina *et al*, 2012) addressed the question of whether histaminergic neurons are organized into distinct functional circuits impinging on different brain regions: they used the double-probe microdialysis technique in freely moving animals, which provides a powerful means for defining the dynamics regulating histamine release in discrete brain regions. 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, the nucleus basalis magnocellularis (NBM), the nucleus accumbens (NAcc)

or the dorsal striatum. By applying compounds targeting receptors expressed on histamine neurons and measuring histamine output in different brain areas, they demonstrated that the same drug influenced differently the release of histamine from distinct brain regions. Bicuculline, a GABAA-R antagonist, acts directly onto histaminergic neurons to augment cell firing (Haas *et al*, 2008). The same authors (Blandina *et al*, 2012) found that intra-hypothalamic perfusion of bicuculline increased histamine release from the TMN, the NAcc and the prefrontal cortex, but not from the striatum (Giannoni *et al*, 2009; Passani *et al*, 2007). Different subunit composition and stoichiometry of GABAA-Rs among histaminergic neurons (Sergeeva *et al*, 2005; Sergeeva *et al*, 2002) may account for these results. It is worth noting that functional heterogeneity of responses to bicuculline among histaminergic neurons relates to TMN neurons heterogeneity with respect to projection fields. Also, responses to H3R antagonists differentiate histaminergic neurons according to their projection areas. When applied to the rat TMN, H3R antagonists, such as thioperamide or the more recently synthesized, non-imidazole compounds as GSK-189254, invariably augmented histamine release from the TMN, from the prefrontal cortex and from the NBM, but not from the NAcc, nor the striatum (Giannoni *et al*, 2009).

In Blandina's and colleagues work (Blandina *et al*, 2012), thioperamide or GSK-189254 were applied locally through the microdialysis probe to the TMN (Giannoni et al., 2009, 2010), hence histamine output increased as a consequence of both somatic and presynaptic H3-autoreceptors blockade, although the participation of only one component cannot be excluded. Thioperamide or GSK-189254, applied locally into the TMN, significantly increased histamine release also from the prefrontal cortex, and the NBM, but histamine levels remained stable in the dorsal striatum and NAcc (Blandina *et al*, 2012). Increases in the prefrontal cortex and NBM were likely due to discharge potentiation of histamine neurons sending efferents to these regions, in a way similar to the effects of TMN perfusion with prostaglandin E2 (Huang *et al*, 2003), or Orexin-A (Huang *et al*, 2001). On the other hand, the lack of increase in histamine release during TMN perfusion with H3R antagonists observed in the dorsal striatum and NAcc, despite the fact that these brain areas receive histaminergic innervation (Airaksinen *et al*, 1989), indicates that histaminergic neurons projecting to these regions are insensitive to H3R blockade. Blandina and co-workers (Blandina *et al*, 2012) further demonstrated that histaminergic neurons are not a homogenous neuronal population using cannabinoid receptor 1 (CB1) agonists. Administration of methanandamide (mAEA) or ACEA in the TMN facilitated histamine release from the TMN itself, from the NBM and striatum 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 (Airaksinen *et al*, 1989) and the presence of histaminergic receptors (Pillot *et al*, 2002).

Spatial segregation due to probe localization does not explain the lack of response, 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 (Köhler *et al*, 1985). This proximity suggests that histaminergic somata projecting to the striatum and prefrontal cortex had the same exposure to H3R antagonists, but were not affected in the same way. In conclusion, H3R antagonists may discriminate groups of histaminergic neurons impinging on different brain regions, thus suggesting that these neurons are organized into functionally distinct circuits that influence different brain regions, and display selective control mechanisms. In keeping with these results, activation of *c-fos* after GSK189254 administration occurred in cortical areas and the TMN, but not in striatum (Medhurst *et al*, 2007b). Local perfusion with H3R antagonists in the NBM or prefrontal cortex augmented significantly histamine release within these regions, an effect that can be explained by blockade of local H3-autoreceptors (Blandina *et al*, 2012). The same drugs administered locally to the striatum or NAcc did not modify histamine release, thus indicating that the whole somatodendritic domain of histaminergic neurons projecting to these regions is insensitive to H3R antagonists. Accordingly, lesion experiments indicate that the vast majority of H3-Rs in the NAcc and striatum are not associated with histaminergic fibres (Pollard *et al*, 1993), which is consistent with high densities of H3R mRNAs levels in the same regions (Chazot and Hann, 2001) and suggests a postsynaptic localization of these receptors. Indeed, H3R are not restricted to histaminergic neurons (Pollard *et al*, 1993), and they act also as heteroreceptors modulating the release of neurotransmitters such as acetylcholine (ACh), dopamine, glutamate, noradrenaline, or serotonin from brain regions crucial for the maintenance of alertness or the storage of information (Haas *et al*, 2008; Passani *et al*, 2007). Although it is generally assumed that all histaminergic neurons express H3R, several isoforms displaying strong differences have been described (Bongers *et al*, 2007). Hence, *in vivo* insensitivity to H3R antagonists may depend on high expression of particular isoforms. In this regard, another observation is intriguing. The distribution of H3R on TMN neurons was examined by performing double immunofluorescence labeling with a combination of anti-H3-R and anti-HDC antibodies (Giannoni *et al*, 2009). Polyclonal H3R antibodies are directed against residues 349–358 of human and rat H3-R and were previously validated (Chazot *et al*, 2001). The density of H3-R immunolabeling was very high in the cytoplasm and on cell membranes of some HDC-positive cell and very low in others, as revealed by confocal microscopy (Cenni *et al*, 2006). A quantitative analysis was carried out counting the

number of pixels with colocalized signal on randomly chosen neurons in hypothalamic brain sections from three rat brains. Setting at 20 the optical density expressed in arbitrary units, two histaminergic neuronal populations that differed significantly for H3R expression levels were found (Blandina *et al*, 2012).

Cotransmitters

Other transmitters or their enzymes are expressed in histaminergic neurons (Kukko-Lukjanov and Panula, 2003). Histaminergic neurons also express galanin, enkephalins, thyrotropin releasing hormone (TRH) and substance P (Haas *et al*, 2008).

Afferent inputs

Behavioural state-dependent activity of histaminergic neurons is modulated by different neuronal, humoral and paracrine signals. The TMN is innervated by the preoptic area in the hypothalamus, the septum, the prefrontal cortex, the subiculum and the dorsal tegmentum (Ericson *et al*, 1991a; Wouterlood *et al*, 1988a; Wouterlood *et al*, 1988b; Wouterlood *et al*, 1987; Wouterlood and Tuinhof, 1992). Inhibitory and excitatory signals arise from the diagonal band of Broca, the preoptic area and the anterolateral hypothalamus, suggesting the release of GABAergic afferents fibres (Yang *et al*, 1997). Monoaminergic and peptidergic afferents are also involved in modulation of histaminergic neurons (Eriksson *et al*, 2004; Sakai *et al*, 1990; Stevens *et al*, 2004; Vorobjev *et al*, 2003).

- Amino acids

  Glutamate: glutamatergic fibres from cortex and hypothalamus are present and glutamate excite TMN that expresses both AMPA and NMDA receptors (Yang *et al*, 1997). The glutamate neuronal transporter EAAC1 was found by using immunohistochemistry near histamine neurons (Faucard *et al*, 2006).

  Glycine and taurine: glycine inhibits a subpopulation of histaminergic neurons (Sergeeva *et al*, 2001), but the presence of glycine fibres in posterior hypothalamus is still uncertain. Taurine, an osmolyte, gates glycine receptors and $GABA_A$ receptors and distribution of this molecule and its transporter are present on histaminergic neurons (Haas *et al*, 2008).

  GABA: GABAergic fibres come mostly from hypothalamic areas and are essential in modulation of sleep/awake cycle (in particular inputs from the ventrolateral preoptic area), firing during sleep, thus inhibiting histamine neurons firing (Ericson *et al*, 1991b; Saper *et al*, 2005; Sherin *et al*, 1998; Steininger *et al*, 2001). The sedative component of general anaesthetics is attributed to the action of GABAergic fibres to the TMN (Sergeeva *et al*,

2005).

- Biogenic amines

  Aminergic and cholinergic neurons send afferents to the TMN: they are excitatory via different mechanisms. Histamine inhibits histaminergic neurons themselves via H3-autoreceptors that are constitutively active (Arrang *et al*, 2007; Gbahou *et al*, 2003; Morisset *et al*, 2000).

  Acetylcholine: nicotinic fast desensitizing action occurs through α7-type receptors (Uteshev and Knot, 2005; Uteshev *et al*, 1996) and represents a sensor for central waking actions of nicotine. Choline has been put forward as the natural ligand in the TMN (Uteshev *et al*, 2005; Uteshev *et al*, 2003). Pharmacological modulation of histamine release by M1 or M3 heteroreceptors in vivo occurs presumably on histaminergic axons (Prast *et al*, 1992).

  Catecholamines: The histaminergic neurons are indirectly regulated by noradrenergic inputs, including from the locus coeruleus. Norepinephrine does not control directly histamine neurons but mediates GABAergic inputs, via an inhibitory action, through α2-receptors (Stevens *et al*, 2004).

  Serotonin: Serotonin stimulates histamine neurons in the rat via the activation of Na^{+}/Ca^{2+} exchange [NCX; (Eriksson *et al*, 2001; Sergeeva, 2007; Sergeeva *et al*, 2003a)].

- Purines (nucleotides, nucleosides)

  Nucleotides excite histaminergic neurons through ionotropic and metabotropic receptors; ATP induces fast non-desensitizing inward currents in TMN neurons and P2X2 represent the major receptor type that occurs in the TMN (Vorobjev *et al*, 2003). ATP, ADP, UTP and 2MeSATP excite TMN neurons via metabotropic receptors, mainly P2Y1 and P2Y4 (Sergeeva *et al*, 2006). Adenosine, that inhibits many types of neurons, has no effect on the TMN (Sergeeva *et al*, 2006), but histaminergic neurons expression adenosine deaminase, an observation that lead to the suggestion that TMN may also use adenosine as a co-transmitter (Selbach, 2007).

- Peptides

  Lots of peptides act as signalling molecules in the hypothalamus where they are involved in endocrine and homeostatic functions.

  Galanin: Galanin is co-expressed in histaminergic neurons of rodents (Airaksinen *et al*, 1992; Kukko-Lukjanov *et al*, 2003; Köhler *et al*, 1986), but not in humans (Trottier *et al*, 2002).

  Orexin/Hypocretin: neurons containing these substances are located next to histamine

neurons and represent a functional entity. Degeneration of hypocretin neurons causes in most cases narcolepsy (Siegel and Boehmer, 2006), because hypocretins maintain wakefulness, particularly in metabolic changes (Sherin *et al*, 1998). Both hypocretins A and B excite histaminergic neurons (Eriksson *et al*, 2000, 2001). Hypocretin neurons also express dynorphin, which can excite histaminergic neurons by suppressing inhibitory GABAergic inputs. The effect of hypocretin in the regulation of vigilance and food intake requires H1R activation; as a matter of fact H1-KO mice present lower hypocretin levels (Lin *et al*, 2002). Corticotropin releasing hormone, glucagon-like peptide-1, neuropeptide Y, ghrelin, thyrotropin releasing hormone: data suggest that TMN controls food intake acting in synergy with leptin: although leptin has no direct effect on TMN, there are secondary targets of leptin that can act on brain and can interact with the central histaminergic system (Toftegaard *et al*, 2003). Several studies have shown the interaction between glucagon-like peptide-1 (GLP-1), corticotropin releasing hormone (CRH) and histamine [reviewed in (Passani *et al*, 2011)]: histamine neurons mediate the GLP-1 induced suppression of feeding, CRH mediates GLP-1 signalling to neuronal histamine and the functional link from GLP-1 to neuronal histamine via CRH constitutes the leptin-signalling pathway regulating feeding behaviour (Gotoh *et al*, 2005). Neuropeptide Y (NPY) fibres are located in the proximity of histaminergic neurons (Tamiya *et al*, 1989) and indirectly affect histamine release (Ishizuka *et al*, 2006). Ghrelin inhibits potassium channels in cultured TMN neurons (Bajic *et al*, 2004). Thyrotrpin releasing hormone (TRH) reduced food intake (Gotoh *et al*, 2007) and sleeping time in rats (Riehl *et al*, 2000). The majority of TMN neurons are excited by TRH (Sergeeva, 2007).

Nociceptin, dynorphin and substance P. Nociceptin occurs in many fibres in proximity of the histaminergic somata in the TMN and strongly inhibits these neurons at postsynaptic level. Morphine, a μ-R agonist, inhibits GABAergic inputs to the TMN exciting histaminergic neurons (Eriksson *et al*, 2000). The κ-agonist dynorphin has no effect, while substance P-positive cells make contact with the somata, the synaptic spines and dendrites of histaminergic neurons (Tamiya *et al*, 1990).

- Metabolic signals (glucose, lipids, CO_2)

  Insulin-induced hypoglycaemia activates TMN in E4 and E5 sub-regions (Miklós *et al*, 2003). Mice that are ApoE-deficient, also express decreased histamine levels and reduced histamine in the amygdala that could contribute to an increased anxiety (van Meer *et al*, 2007). Estrogen receptors are expressed in human TMN and their levels change metabolic

activity, sex, aging and are involved in Alzheimer's disease (Verdière *et al*, 1977). Prostaglandin E2 activates TMN to induce wakefulness in rats (Huang *et al*, 2003). Endocannabinoids increase histamine release selectively in the TMN (Cenni *et al*, 2006). TMN histaminergic neurons could also be involved in CO1-mediated arousal (Johnson *et al*, 2005).

Histaminergic pathways and targets

Similar projection scheme of histaminergic fibres has been found between species but differences exist in the density of the innervation of the specific area (Haas *et al*, 2008). In rodents, arborizing axons reach the entire central nervous system via two ascending and one descending pathways (Köhler *et al*, 1985; Panula *et al*, 1990; Smits *et al*, 1990; Watanabe *et al*, 1984; Wouterlood *et al*, 1986). One ascending pathway travels in the ventral surface of the median eminence to the hypothalamus, the diagonal band the septum and olfactory bulb, the hippocampus and cortex, the other leaves the TMN dorsally and runs along the third ventricle to the thalamus, basal ganglia, hippocampus, amygdala and cortex. The descending goes to the brain stem. No correlation seems to be present between the specific TMN somata and their projections. Tracing studies describe how histaminergic fibres are strongly crossing and highlighted that many neurons make contacts with more than one of the initial pathways (Hayashi *et al*, 1984; Panula *et al*, 1984).

The highest density of histamine fibres has been described in the hypothalamus. The septal nuclei receive strong histaminergic innervation. The ventral tegmentum and the dopaminergic nuclei instead receive a moderate to dense histaminergic input. Histaminergic fibres entre the hippocampus via both anterior and posterior pathways and reach moderate densities. Moderate densities are present as well in the amygdala (Haas *et al*, 2008).

The tuberomamillary nucleus as an integrative centre: heterogeneous functions of histamine in the central nervous system

The widespread distribution of histaminergic projections throughout the brain allows histamine to control several homeostatic, behavioural and pathological conditions. In particular, histamine controls arousal during wakefulness (Takahashi *et al*, 2006). Central histamine is mainly involved in qualitative and cognitive aspects of vigilance (Anaclet *et al*, 2009), coordinates goal-directed behaviours, like food provision (Valdés *et al*, 2010) and modulates mnemonic processes, in a temporal specific manner according to the type of memory and brain areas involved (Passani *et al*, 2007). Using retrograde tracers to map histamine projections in different brain areas, it appeared

that histaminergic neurons do not show a topographic organization nor a topographical scheme (Köhler *et al*, 1985); however, it is now evident that histaminergic neurons are organized in distinct and diverse functioning units, which enable different drugs and ligands to influence signalling in different parts of the brain (Passani *et al*, 2011). The full implications and therapeutic importance of the functional heterogeneity of histaminergic cells in the central nervous system can be better evaluated considering and studying the differentiated regulation, in a region-specific way, of neurotransmitters that are known as main regulators of cognitive functions, motivated behaviour and behaviour processes (Passani *et al*, 2011).

Modulation of behaviour by the central histaminergic system: lesson from H1-, H2, H3 and HDC-KO mice

Arousal, learning, memory and emotional behaviour

As already described H1- and H2Rs are strongly expressed in brain areas that are involved in regulating cognitive processes and emotional behaviours, such as hippocampus, amygdala, thalamus, hypothalamus and cortex (Panula and Nuutinen, 2013). Moreover, hippocampus and the amygdaloid nucleus are responsible for the modulation of emotions like anxiety and depression (Walf and Frye, 2006).

H1- and H2-KO mice do not show differences in the performance on rotarod, no differences in depressive-like behaviour, although a decreased number of H1Rs has been detected in the brain of depressive patients by Kano and colleagues (Kano *et al*, 2004), lower levels of anxiety (Yanai *et al*, 1992). These genotypes also present decreased performances in different memory paradigm: a decreased novel object discrimination in conditioned place preference for H1-KO mice (Zlomuzica *et al*, 2008). H1-KO mice are also seriously impaired in temporal order memory [as a model of "global dementia"; (Zlomuzica *et al*, 2013), in spatial memory in the eight-arms maze (Zlomuzica *et al*, 2009) and present impairments in long-term motor activity and episodic-like memory (Dere *et al*, 2008); both H1- and H2-KO mice are characterized by a worse performance in object recognition, spatial learning in the Barnes maze and present a decrease in hippocampal LTP (Dai *et al*, 2007).

As that the H3R acts as an auto- and hetero-receptor and as it is characterized by a high constitutive activity, H3-KO mice provide a genetic tool to study its involvement in different behaviour: these mice present higher levels of anxiety in acoustic startle test but no differences in the open field compared to WT animals, decreased anxiety in the elevated plus maze and zero maze and a better spatial memory (Rizk *et al*, 2004).

HDC-KO mice, animals genetically unable to synthesize histamine, present various changes in behavioural characteristics: they present higher levels of anxiety and a better retention of emotional memory (Acevedo *et al*, 2006a), no differences in novel object or novel location recognition (Acevedo *et al*, 2006b) and a worse performance in non-reinforced relational object memory task (Dere *et al*, 2008).

Regulation of energy expenditure and food intake

Histaminergic neurons densely innervate hypothalamic areas critically involved in the regulation of food intake and body temperature (Panula *et al*, 2013) and interact with other neurotransmitters and neuropeptides involved in these homeostatic behaviours and processes (Schneider *et al*, 2015).

H2-R are not involved in modulation of feeding but rather the diuretic action of histamine (Lecklin *et al*, 1998).

H1-KO animals, on the other hand, present differences in feeding behaviour (Masaki *et al*, 2001); no difference in body weight was observed but the effect of leptin was reduced in high fat diet fed animals along with augmentation of body fat and leptin expression (Masaki *et al*, 2001). The basal food intake and NPY expression is enhanced in H1-KO mice, while orexin A is not effective on this genotype (Jørgensen *et al*, 2005), as well as the anorectic effect of TRH (Gotoh *et al*, 2007), nesfatin-1 (Gotoh *et al*, 2013), neurotensin [also in pyrilamine-treated WT mice; (Ohinata *et al*, 2004)] and estrogen (Gotoh *et al*, 2007).

H3-KO mice are instead characterized by a diminished food intake, less alcohol preference and consumption and alcohol-induced conditioned place preference (Nuutinen *et al*, 2010). Mice chronically deprived of histamine present augmented brown adipose tissue, blood insulin and leptin and less glucose tolerance (Fulop *et al*, 2003).

CHAPTER I

BRAIN HISTAMINE AND MEMORY MODULATION

Memory

Memory defines the ability of the brain to acquire, or encode, consolidate, therefore store, and retrieve information (Izquierdo and Medina, 1997). Organisms inherit in the structures of their nervous system many adaptations developed as a result of variation and natural selection operating during previous generations; yet they also inherit the potential to adapt or change as the results of events occurring during their own lifetime. Because of this adaptation, the experiences of an organism can modify the nervous system and the organism later can behave differently because of these experiences. The ability to change gives organisms the capacity for learning and memory (Squire, 1986). Phenomena occurring in life can modify real learning and memory as drug tolerance, synaptic sprouting after a brain lesion, enzyme induction, recovery of function after brain injury and strictly synaptic events. These phenomena all reveal ways in which the brain can change after certain experiences. In 1950, Karl Lashley, a pioneer in studying brain and behaviour, wrote pessimistically about the "problem of memory":

"This series of experiments… has discovered nothing directly of the real nature of the engram. I sometimes fell, in reviewing the evidence on the localization of the memory trace, the necessary conclusion is that learning just is not possible "(Lashley, 1950, p 477-478).

Since then, giant technological growth in neurosciences has made it possible to study the problem of memory.

Phases of memory

Memory, a dynamic cognitive function, consists in a process subdivided in phases: acquisition phase, normally called learning, the translation in the brain of external stimuli or internal representations, like an emotion (Kandel and Squire, 2000; McGaugh, 1966), once translated and acquired the information is consolidated, strengthened and stored into different networks in the brain, with consequent changes in electrophysiological activity and protein synthesis. After consolidation the memory trace, or engram, can be recalled or retrieved, via a recall phase (Izquierdo *et al*, 1997). A memory that has been consolidated can be re-acquired (Sara, 2000), through the re-consolidation process, because during recall the engram becomes labile and susceptible to modifications (Nader *et al*, 2000; Sara, 2000). A memory trace could be also extinguished: extinction is not a sort of passive forgetting, it is the learned inhibition of retrieval of

previously acquired responses (Furini *et al*, 2014), so an active process that could be used, for example as a major component of exposure therapy in the treatment of fear memories, such as those of the post-traumatic stress disorder [PTSD; (Furini *et al*, 2014)].

History of the study of memory

The study of memory is actually traced back to Herman Ebbinghaus (1885/1913, translation), who examined his own acquisition and forgetting of new information in the form of series of nonsense syllables testes at various periods up to 31 days. Among many important observations, he noticed that he often had a "first fleeting grasp…of the series in moments of special concentration" but that this immediate memory did not ensure that the series had been memorized in a way that allows its recall later on. Stable memorization sometimes required further repetitions of the series. Soon afterward, James (1890) proposed a distinction between primary memory, the small amount of information held as the trailing edge of the conscious present, and secondary memory, the vast body of knowledge stores over a lifetime. The primary memory of James is like the first fleeting grasp of Hebbinghaus (Cowan, 2008).

The Industrial Revolution posed new questions on what James called primary memory. Primary memory is called into play with the necessity, for example to fast memorize a telephone number in order to use it in seconds or when someone is asked to keep in mind aspects of unfamiliar situations, such as names, places, things and ideas that one has not encountered before (Cowan, 2008).

The modern era of memory research can be said to have begun in 1957 when Brenda Milner described the profound effects on memory of bilateral medial temporal lobe resection, to relieve from epilepsy a patient who became known as HM (Scoville and Milner, 1957; Squire, 2009). HM exhibited a strong forgetfulness against a background of largely intact intellectual and perceptual functions. The finding from HM patient established three fundamental principles that continue to guide actual experimental work of mnemonic processes. First, memory is a distinct cerebral function, separable from other cognitive abilities. Second, because HM did, as well others of his age retain a number or a visual image for a short time, it was concluded that the medial temporal lobe is not needed for "immediate" memory. Third, the damaged structures in HM were not the ultimate repository of memory, because he retained his remote memories of the childhood.

Efforts to achieve an animal model of human memory impairment succeeded initially in monkey (Mishkin, 1978). Behavioural, together with neuroanatomical studies, begun to identify structures that can modulate memory processes, such as the hippocampus, the entorhinal and

parahippocampal cortices (Squire and Zola-Morgan, 1991). The neuroanatomical studies also identified boundaries and the connectivity between the memory-modulating areas, initially in the monkey and then in the rat (Squire *et al*, 1991; Suzuki and Amaral, 1994).

The possibility of studying specific connections was improved by the use of new genetic and physiological techniques, as well as neuroimaging techniques, which take advantage of the detailed neuroanatomical information (Squire, 2009).

Memory classification

Memories can be classified following different categories: according to their content (Markowitsch, 1997; Squire, 1992), according to their duration (Fuster, 1998; Markowitsch, 1997), and according to their nature: archival as opposed to transient, moment-to-moment (Goldman-Rakic, 1992, 1996). Two of these criteria a mostly use to study memory processes starting from the duration and the content. Regarding the duration Atkinson and Shiffrin (Atkinson, 1968) divided the structure of memory into three components: the sensory register, the short-term store and the long-term store.

The first mnemonic step that an incoming stimulus makes is called sensory memory. All sensory modalities (visual, auditory, tactile, olfactory and kinaesthetic) are involved in receiving first impressions, which could be either lost or processed further for later remembering. The duration of sensory memories is very short and it varies in time for different sensory modalities: visual sensory memory (iconic memory) may last from 250 msec up to 500 msec; auditory sensory memory (echonic memory) from 2 to 10 sec; tactile memory about four and motor memory (kinaesthetic) as long as 80 sec (Aman, 1986).

Short-term memory (STM) consists in the acquisition of sensory information and it was clearly distinct from long-term memory when it was found that individuals with hippocampal lesions may have an intact STM, but present severe impairment of LTM (Scoville *et al*, 1957). An item can be remembered for minutes or hours, and this memory can, eventually, via the consolidation process, be converted in long-term memory (LTM), which is characterized by a retention of days, weeks or even years (Izquierdo and McGaugh, 2000; McGaugh and Izquierdo, 2000). During acquisition and formation of the STM the synaptic connection is strengthened by cascades of processes that comprehend the activation of receptors, but without protein synthesis (Kandel, 2001). The consolidation of information into LTM instead needs the synthesis of new proteins with new neuronal circuits (Furini *et al*, 2013).

In between STM and LTM, Miller in 1960 defined another type of memory, the working memory that allow us to remember an information only for the time sufficient to elaborate a thought or to do an action, for example allow us to remember a telephone number only to be able to digit it (Izquierdo *et al*, 1999).

Depending on the content LTM could be divided in declarative (or explicit) memory and non-declarative (or implicit) memory: declarative memory provides a way to represent the external world, it is the kind of memory we typically have in mind when we use the term memory in everyday language. Declarative memory can be further divided in two components: semantic memory, about facts, and episodic memory, which is the ability to re-experience a time-and-place specific event in its original contest (Squire, 2004). Declarative memory is well adapted for the rapid learning of specific events and allows remembered material to be compared and contrasted, with consciousness: as a matter of fact, the stored representations are flexible, accessible to awareness and can guide performance in different contexts (Squire, 2004). The keys structures that control declarative memories are the hippocampus, the entorhinal, perirhinal and parahippocampal cortice (Squire *et al*, 1991). These structures are organized hierarchically and their anatomy suggests how every structure may contribute differently to the formation of declarative memory, for example in encoding object (perirhinal cortex) or scenes (parahippocampal cortex) and in forming associations between them [hippocampus; (Davachi, 2006; Squire, 2004; Staresina and Davachi, 2006)].

Non-declarative memory is dispositional and is expressed through performance rather than recollection. An important principle is the ability to gradually extract the common elements from a series of separate events; this subtype of LTM provides for a myriad of unconscious ways of responding to the world. Non-declarative memory refers to a collection of abilities that are expressed throughout performance without conscious memory content. Characteristic of this type of memory is priming, that is the ability to improve access to items that have been recently presented or improve access to associates to those items (Squire, 2004). Priming is presumably advantageous because animals evolved in a world where things that are encountered once are likely to be encountered again: priming improves the speed and the efficiency with which organisms interact with a familiar environment and may influence feature-based “attentional” processes (Hutchinson and Turk-Browne, 2012; Theeuwes and Van der Burg, 2013).

Another early example of non-declarative memory is simple classical conditioning, best illustrated in the literature by the delay eye blink conditioning. In delay conditioning, a neutral conditioned stimulus (CS), such as a tone, is presented just before an unconditioned stimulus (US), such as an

air puff to the eye. The two stimuli then overlap and co-terminate. Critically, delay eye blink conditioning is intact in amnesia and is acquired independently of awareness (Clark and Squire, 1998; Gabrieli *et al*, 1995). Participants who did not become aware of the relationship between the CS and US (i.e., that the CS predicts the US) learned just as well as volunteers who did become aware (Clark *et al*, 2001; Manns *et al*, 2001). Indeed, when CS–US association strength was varied (by changing the number of consecutive CS alone or CS–US presentations), the probability of a conditioned response increased with association strength but was inversely related to how much the US was expected (Clark *et al*, 2001). Largely on the basis of work with rabbits, delayed eye blink conditioning proved to depend on the cerebellum and associated brain stem circuitry (Thompson and Krupa, 1994; Thompson and Steinmetz, 2009). Forebrain structures are not necessary for acquisition or retention of classically conditioned eye blink responses. Evaluative information, that is, whether a stimulus has positive or negative value, is acquired largely as non-declarative memory. Studies of this kind of memory have focused especially on the associative learning of fear (Adolphs and Anderson, 2013; Davis and Davis, 2006; LeDoux, 2014), which is an acquired behaviour after threatening events. Its non-declarative status is illustrated by the fact that, in humans, associative fear learning proceeded normally after hippocampal lesions, even though the CS–US pairings could not be reported (Bechara *et al*, 1995).

Fear memories, classical conditioning and fear conditioning

All organisms, even single cell organisms, must have the capacity to detect and respond to significant stimuli in order to survive (Macnab and Koshland, 1972). With the evolution of multicellular organisms with specialized systems, particularly a nervous system, the ability to detect and respond to significant events increases in sophistication (Shepherd, 1983). The ability of processing threats that lead to fear is essential for survival and its memory is as well. The term “fear” refers to a subjective feeling state and to the behavioural and physiological responses and because fear plays a prominent role, directly or indirectly, in a variety of psychiatric conditions understanding its neuronal basis is of great importance (Johansen *et al*, 2015).

Pavlov demonstrated that dogs could learn to use a neutral cue to predict a biologically relevant event via what was later called “classical conditioning paradigm” (VanElzakker *et al*, 2014). Briefly Ivan Pavlov rang a bell (the conditioned stimulus) and immediately after he presented food to the dog [the unconditioned stimulus; Pavlov, 1927; (VanElzakker *et al*, 2014)]. On its own the food made the dog salivate (the unconditioned response, UR). After repeating this predictive pairing several times, the dog began salivating to the mere sound of the bell, even if no meat was

presented, making salivation the conditioned response (CR). This ability in nature is essential because a stimulus, that does not have a specific meaning for the animal, such as the sound of the bell, predicts something agreeable and biologically valuable: food (VanElzakker *et al*, 2014). However, not all Pavlov's USs were pleasant and not all CRs conveyed the dogs anticipation of something enjoyable as it is important for organisms to be able to predict and anticipate threats to health and safety. For example, when Pavlov repeatedly paired the sound of a metronome (CS) with subsequent application of a sour-tasting diluted acid (US) onto the dog's tongue, the dog eventually learned the association. Henceforth, upon presentation of the CS alone, the dog exhibited what Pavlov called a "defensive reflex": it shook its head, salivated profusely, and moved its tongue as if to expel a toxic substance, even though no acid was there. A similar process was demonstrated with an 11-month-old child in Watson and Rayner's famous "Little Albert" experiments of 1920. Watson and Rayner paired Albert's touching of a white rat (CS) with a sudden fear-arousing noise (US) made by striking a steel bar behind him (Watson and Rayner, 2000). Upon subsequent presentations of the rat, Albert no longer exhibited his natural curiosity, but rather withdrew his hand. This learned response seemed to generalize to cotton balls, a Santa Claus mask, a brown bunny, and a black fur coat. The Little Albert experiment is an early precursor of what is now known as fear conditioning.

Evaluation of fear memories in rodents

When rodents sense danger, one species-specific behavioural response is to freeze all movement in order to avoid detection by predators. Rodent fear conditioning and extinction studies typically use a foot shock as the US. The fear response is evaluated as the percentage of time a rodent spends engaging in freezing behaviour. When a light or tone (CS) repeatedly predicts a foot shock (US) delivered through an electrified metal cage floor, rodents are conditioned to make a CS-US association. Thus, the presence of the CS subsequently triggers freezing, which becomes the CR. Furthermore, when a rodent experiences an aversive US such as shock in a certain context, subsequent re-exposure to that context can cause freezing behaviour in the absence of the shock. This type of Pavlovian fear conditioning is known as contextual fear conditioning (Rudy *et al*, 2004). When a rodent experiences a sudden loud noise it will startle before freezing, but if that sudden loud noise occurs during the presentation of a danger-associated cue such as a CS or a conditioning context, the startle reflex will be larger. This is known as a fear-potentiated startle and is another commonly evoked CR (Davis, 2001). The fear-potentiated startle paradigm is advantageous for translational research because it is not species-specific.

Researchers can link conditioned behaviours such as freezing or fear-potentiated startle to brain

activity or other fear-based physiological measures. The sensory experiences of the CS and US are processed in the thalamus and somatosensory cortex, as are other sensory experiences. This information reaches the lateral amygdala via one of two routes. A “cortical pathway” relays detailed sensory information through the thalamus to the neocortex and hippocampus before integration and evaluation in the lateral amygdala. However, another pathway forgoes the neocortex in the service of reaction speed. This faster “subcortical pathway” projects a rudimentary sensory representation directly from the thalamus to the lateral and central nuclei of the amygdala. The binding together of a conditioned CS-US association is supported by the lateral nucleus of the amygdala, which then projects to the central amygdala, triggering autonomic and behavioural responses such as freezing (Blair *et al*, 2001) and fear-potentiated startle (Campeau and Davis, 1995). The amygdala is part of a broader neuro-circuitry that supports and modulates this process. Conditioning is modulated by medial prefrontal cortex (mPFC) structures. The more dorsal prelimbic cortex of the rodent is associated with the expression of conditioned fear (Burgos-Robles *et al*, 2009). The prelimbic cortex acts as a fear response “accelerator” during conditioning, while the more ventral infralimbic cortex acts as “brakes” during extinction. The infralimbic cortex is necessary for fear conditioning responses to context (Resstel *et al*, 2006), probably due to its connectivity to hippocampus and amygdala (Bouton *et al*, 2006; Maren *et al*, 2013). The hippocampus serves the function of binding together the disparate sensory and interoceptive elements that form a context into one conjunctive representation (O'Reilly and Rudy, 2001). The rodent hippocampus has connections with both prelimbic and infralimbic cortex and thus provides contextual modulation over fear responses. Furthermore, during exploration of the environment, the hippocampus, along with associated medial temporal cortex, serves as a functional comparator of present and past, stored, experience (VanElzakker *et al*, 2008). As such, it is vital to the recognition of a context as familiar or the establishment of a context as novel. A related function is its involvement in comparing novel cues to an existing CS, to determine if a CR is appropriate; stimulus generalization is what led Little Albert to be wary of cues that only moderately resembled a white rat. The hippocampus is therefore a crucial structure in determining whether contextual cues are associated with danger or with safety (Maren *et al*, 2013; Rudy *et al*, 2004). There exist other behavioural tasks that help scientists to evaluate fear memories in rodents such as Inhibitory Avoidance (IA) that involves learning to inhibit a response in order to avoid an aversive stimulus. The learning (training) session may consist of one-trial or multi-trials. Since a punishment follows the natural exploratory drive of a rodent with a non-letal, pulsating electric footshock, this is clearly an *aversive* task. IA involves both an explicit, associative component (to the context), and

an operant-like conditioning component (to the shock, this last being considered a type of implicit memory). There are two different approaches to the IA behaviour, the step-down IA, here described in more detail, and the step-through IA [see, e.g., (Bermúdez-Rattoni *et al*, 1997)]. In the Two-Way Active Avoidance (AA) the animal learns that a random stimulus (a tone, the CS) is a reliable predictor for a coming aversive experience (a shock, the US), and can prompt an evasive action in order to avoid it, i.e., it moves to the other side of the shuttle box (the CR) when the stimuli predict aversive events. Since there is the possibility to learn how to escape, this task may be classified as an operant (or instrumental) conditioning, i.e., the animal must learn the relation between CS (sound) and US (shock) in order to anticipate US with a CR (escape) and avoid it. This task is also called Shuttle Avoidance, in reference to the strategy the animal must learn and perform (Diehl *et al*, 2007).

Central histamine in mnemonic processes

Histamine in synaptic plasticity

Long-term potentiation (LTP) is considered the main demonstration of synaptic plasticity. It was first described in the hippocampus, a brain region directly involved in the formation of several memory types (Bliss and Lomo, 1973; Izquierdo *et al*, 1997; Scoville *et al*, 1957). It consists of the long lasting enhancement of the postsynaptic response following a high-frequency afferent stimulation. This is seen as an increase in the excitatory postsynaptic potential evoked by a single stimulation pulse when compared to the response evoked before the high-frequency stimulation (Bliss *et al*, 1973). There is evidence that this form of synaptic enhancement is also present in other regions of the cerebral cortex, especially during early developmental stages (Lynch, 2004). LTP has several characteristics that make it a strong candidate for the cellular mechanism responsible for long-term memory storage (Lynch, 2004).

Among the several mechanisms that are jointly responsible for LTP, the activation of the glutamatergic NMDA receptor plays a central role in both the induction and maintenance of most LTP types (Lynch, 2004). The calcium influx in the postsynaptic dendritic spine leads to the activation of several signalling cascades, notably the calcium/calmodulin-dependent protein kinase II (CaMKII), the protein kinase A (PKA), the protein kinase C (PKC), and the mitogen-activated protein kinase (MAPK). Their final course of action is the activation of transcription factors like CREB, which controls the synthesis of the proteins required for the maintenance of the increased postsynaptic response (Lynch, 2004; Sweatt, 1999). This process is modulated by histamine (Dringenberg and Kuo, 2006), together with other neurotransmitters. LTP was facilitated by

histamine in the CA1 area of rat hippocampal slices, since it could be induced with a weak tetanic stimulation when histamine was added to the bath, and this effect persisted even in the presence of H1- and H2R antagonists. It was then demonstrated that histamine or its main metabolite, 1-methylhistamine (Williams *et al*, 2006), may enhance NMDA induced response and subsequently hippocampal LTP by means of direct channel activation by binding to the polyamine site (Haas and Panula, 2003; Vorobjev *et al*, 1993).

Regarding the effects of histamine on LTP, H1R activation has been shown to reduce the blockade of the NMDA receptor channel mediated by Mg^{2+}, by activating PKC, which leads to increased activation of the glutamatergic receptor (Payne and Neuman, 1997). In addition, as already pointed out, histamine can also directly activate the NMDA receptor by binding to the polyamine modulatory site present in this receptor (Dai *et al*, 2007). Binding is pH sensitive and is restricted to the NR1/NR2B subunits of the NMDA receptor (Haas *et al*, 2008). Furthermore, H1R activation leads to the synthesis of postsynaptic retrograde messengers, such as nitric oxide and arachidonic acid, which might be responsible for presynaptic modifications that occur after LTP induction (Haas *et al*, 2003). As an example of the involvement of this receptor in LTP, H1 receptor knockout mice showed impaired LTP induction in the CA1 region of the hippocampus (Dai *et al*, 2007).

As mentioned above, the H2 receptor is a potent stimulator of the cAMP-signalling pathway, which is necessary for the late phase of NMDA receptor-dependent LTP (Frey *et al*, 1993) and for LTP expression in giant hippocampal mossy fibre synapses (Weisskopf *et al*, 1993). H2Rs can also increase ionic currents generated by the activation of the NMDA receptor, through blockade of calcium-dependent potassium channels (Haas, 1974), and possibly by mediating the phosphorylation of the NMDA receptor itself by PKA (Raman *et al*, 1996). LTP induction in the hippocampus was also impaired in H2 receptor knockout mice (Dai *et al*, 2007).

The effects might change across different brain regions. H3Rs can reduce the release of several neurotransmitters, including glutamate, which might affect synaptic plasticity in hippocampus and striatum (Brown and Haas, 1999). In dentate gyrus, for instance, H3R activation hinders synaptic transmission and reduces paired-pulsed facilitation, which is a short-term form of synaptic plasticity (Brown *et al*, 1996). In the CA3 region of the hippocampus, histamine promotes synchronized bursts of action potentials (Yanovsky and Haas, 1998), an activity pattern that is known to be a physiological stimulus for the occurrence of LTP in the CA1 region (Bliss and Collingridge, 1993). In the CA1 region, when Ca^{2+} levels are low and Mg^{2+} levels are high, histamine causes a lasting potentiation of neuronal excitability, which is mediated mainly by H2 receptor activation and the PKA signalling pathway, with H1 and NMDA receptors having a modulatory role

in that downstream cascade.

Thus, histamine can enhance LTP through at least three different mechanisms: H1Rs favour LTP by increasing intracellular levels of Ca^{2+} and subsequent PKC activation, both required for LTP induction; the effects of the H2Rs are mediated by cAMP/PKA signaling pathway activation, which is involved in the maintenance of LTP, and finally, the NMDA receptor can be directly activated by histamine binding to the polyamine site, which can modulate both LTP induction and maintenance (Brown *et al*, 1996; Haas *et al*, 2003; Vorobjev *et al*, 1993).

Histamine and Memory

Antihistamines are frequently part of the treatment regimen for seasonal and perennial allergic rhinitis occurring alone or in conjunction with associated airway disorders, such as asthma, sinusitis, and otitis media. These agents are also frequently prescribed for the treatment of urticaria to eliminate the need for longterm corticosteroids. Sedating antihistamines cross the blood-brain barrier more quickly and easily than the nonsedating antihistamines, they produce more central nervous system (CNS) effects, exacerbating the decreases in decision-making, verbal learning, and psychomotor skills already experienced by the patient with allergic rhinitis (Nolen, 1997).

Performance in the classroom is adversely affected by sedating antihistamines. A study conducted in the Netherlands involving 52 primary-school children with allergy and 21 healthy students demonstrated that learning was impaired in atopic patients compared with healthy controls (Vuurman *et al*, 2014). Furthermore, treatment with the sedating antihistamine diphenhydramine impaired learning to an even greater degree than the atopy itself. Learning was assessed using computer simulated role-playing activities that required children to manage farmland in North Africa while dealing with variables such as weather. Results of this study confirm that learning performance is negatively impacted by the onset of allergic rhinitis and further compromised by treatment with sedating antihistamines (Nolen, 1997).

Systemic Administration of Histamine and Memory Performance.

There is a considerable body of work on the role of histamine in models of memory impairment induced by systemic administration of antagonists of the neurotransmitters acetylcholine and glutamate, most notably using the muscarinic receptor antagonist scopolamine or the NMDA receptor antagonist MK-801. The reversal of memory deficit produced by systemic administration of histamine in these models of memory impairment was evaluated in several behavioural tasks.

The evidence suggests that this reversal comes about as a result of the interplay between the

histaminergic and cholinergic systems. It is known that both cholinergic and GABAergic projections of the septum to the hippocampus are important in generating hippocampal theta rhythm (Bassant *et al*, 1995; Lee *et al*, 1994). It has been demonstrated that histamine can activate GABAergic neurons in the septohippocampal pathway, by binding to the H1- and H2Rs (Xu *et al*, 2004). Furthermore, electrical stimulation of TMN has been shown to increase acetylcholine (ACh) release in the hippocampus (Mochizuki *et al*, 1992), and intraseptal infusions of histamine also enhance hippocampal ACh release (Bacciottini *et al*, 2002). Thus, histamine can regulate the hippocampal electrical activity and induce ACh release in the hippocampus in physiological states. The improvement seen in scopolamine-induced deficits might be mediated by such modulation of the septohippocampal pathway (Köhler, 2011).

Systemic studies that investigate the impact that histamine has on memory in a physiological context show the effect to differ with respect to the behavioural paradigm, and thus the memory type, and most studies, deals with the direct manipulation of a single histamine receptor. In aversive tasks like inhibitory avoidance, the systemic administration of an H3R antagonist improves performance. These studies have used ligands with varying degrees of potency, bioavailability, and affinity for studying species receptor (Lim *et al*, 2005). While some studies used classical ligands now classified as inverse agonists like thioperamide and clobenpropit, which have nonspecific actions, notably on the 5-HT3 serotonergic receptor and α_2C adrenergic receptor; (Lim *et al*, 2005)], studies employing ligands with a different chemical structure and with more selectivity and affinity to the H3R showed similar memory enhancing effects. By contrast, the administration of an H3R agonist impairs performance (Blandina et al., 1996), and both non-specific ligands like RAMH (also active on noradrenergic receptors) and imetit (also active on serotonergic receptors) and the more selective ligand immepip produced similar memory impairing effects. Together, these data highlight the possible role of H3R-mediated modulation on aversive learning.

On the other hand, the administration of an H1R antagonist has produced opposite results for aversive memory tasks (Frisch *et al*, 1997; Kamei *et al*, 1990). It must be noted, though, that these studies used experimental subjects that differed in age. The expression pattern of the histamine receptor subtypes changes with age in rodents. Since the H1R shows the most consistent changes in density (Terao *et al*, 2004), this might account for the opposite effect. Finally, the alkylamine chlorpheniramine used in one study also has nonspecific actions, notably the inhibition of noradrenaline and serotonin reuptake, which could also account for the reported effects (Niemegeers and Leysen, 1982). There are some differences between passive and active avoidance tasks regarding learning, as active avoidance involves a multi-trial learning task, while passive

avoidance is a single-trial task, therefore more suitable to pharmacologically manipulation. The only study that evaluated first generation and the more selective second generation H1 blockers using active avoidance showed that other variables might indeed play a role in the evaluation of the action of H1 receptors on aversive memory (e.g. rat age): although all the compounds impaired learning, the extent of the impairment seems to be greater for the nonspecific compounds (Frisch *et al*, 1997).

The results of systemic studies are contradictory regarding the effect the histaminergic system has on spatial memory tasks such as the water maze. The administration of a histamine precursor did not produce any effects, while the administration of an H3R agonist improved performance (Rubio *et al*, 2008). This may be explained by the fact that the H3R is also found on neurons that release other neurotransmitters, thus improvement could be mediated by the regulation of other systems involved in spatial learning, such as the dopaminergic system (Granado *et al*, 2008; Mura and Feldon, 2003), and not only by increasing the synaptic availability of histamine. Another explanation would be that the H3R RAMH ligand is not specific to histamine and may also have affinity for other aminergic receptors (Lim *et al*, 2005).

Knockout mice models and memory performance

Recent studies have used transgenic models in the attempt to identify the role of histamine and each receptor subtype on memory processes.

There is evidence that the lack of the histidinedecarboxylase enzyme (HDC) produces changes in the performance of some memory tasks, depending on the memory type being addressed. In the water maze task, spatial memory has been shown to be both hindered and improved in histidinedecarboxylase knockout mice [HDC-KO mice; (Acevedo *et al*, 2006a; Dere *et al*, 2008)] though these differences may be influenced by the gender used in the models.

Knockout models for H1- and H2Rs, on the other hand, have shed more light on the physiological role of histamine in learning. They act negatively on the learning of aversive tasks but facilitate spatial and discriminative ones. Interestingly, the only study that addresses the role of the three histamine receptors in a Barnes-maze task showed that the lack of the H3 receptor improves memory. The absence of presynaptic auto- and hetero-receptors might potentiate the action of histamine in postsynaptic H1- and H2Rs, which also modulated the memory associated with this particular task.

An important factor in the interpretation of studies with knockout models is that the inactivation of the genes of interest is not restricted to an area or specific part of the circuits involved, and thus, these effects are likely to reflect the overall action of histamine or its receptors. Studies using

temporally and spatially restricted knockout models would clarify the role of histamine in each

Intracerebrovascular Administration of Histamine and Memory Performance

The administration of histamine or its receptor agonists/antagonists in the ventricular system is a more convenient way to study the role of histamine in the CNS than peripheral administration, due to BBB permeability and potential peripheral side effects that may confound the outcome.

Histamine has facilitatory actions in social learning, since its i.c.v. infusion improved, while the inhibition of its synthesis compromised the performance. Also, pharmacological stimulation with the potent and selective H3R agonist immepip led to impaired performance in learning the task, while the use of the inverse agonist thioperamide facilitated it. The role of the other receptor types is not addressed, and thus, the H3R response and histamine effect might be due to the regulation of other neurotransmitter systems (Tabella per ref)

Conversely, findings of studies that used tasks based on learning an aversive memory are controversial. Studies that employed a passive avoidance task showed either improvement or impairment of the task following i.c.v. administration of histamine (de Almeida and Izquierdo, 1986, 1988; Kamei *et al*, 1990; Zarrindast *et al*, 2002; Zarrindast *et al*, 2008), but the doses administered varied: 1–10 ng/rat of histamine improved performance, while 20 μg/rat impaired it. The EC50 of the H3Rs for histamine is at least one order of magnitude lower than H1 and H2 receptors (Brown *et al*, 2001b), and thus, the low concentration might be causing a mainly H3-based response, thus acting negatively on the histaminergic system, which would explain the reported improvement in aversive memory. Since H1- and H2Rs blockade also improved the memory for the same task, this would corroborate the inhibitory actions of histamine in aversive learning (Zarrindast *et al*, 2002; Zarrindast *et al*, 2008).

The findings of two studies employing the active avoidance paradigm were also contradictory (Chen *et al*, 1999; Kamei *et al*, 1990; Nishiga and Kamei, 2003b; Prast *et al*, 1996), but the study that showed memory improvement was found with the use of aged rats, which might account for the reported contradictory effects. Finally, there is controversy regarding the role of the H1R in the active avoidance task, since it was shown that this could either inhibit or facilitate performance in active avoidance tasks(Chen *et al*, 1999; Prast *et al*, 1996).

The i.c.v. administration of compounds that act on the histaminergic system is less studied in spatial memory and object recognition or discriminative memory types. Histamine reverses the impairment induced by NMDA antagonists in a radial maze task (Nishiga *et al*, 2003a). The activation of both glycine and polyamine sites can act synergistically in memory function (Kishi *et*

al, 1998), and thus, histamine might compensate the deficit through direct action on the NMDA receptor. However, in a physiological context, the blockage of histamine synthesis (Chen *et al*, 1999) or lack of the gene that encodes the H1R impaired performance in the radial maze (Zlomuzica *et al*, 2009), while H1R inactivation improved the memory in the water-maze task (Hasenöhrl *et al*, 1999). Methodological issues of these tasks that rely on spatial memory might explain this differential effect. H1R activation facilitated learning the object recognition task, and this is in agreement with studies that used knockout models (Tabella). The brain areas involved are slightly different for spatial and discriminative tasks, with spatial memory depending mainly on the hippocampus, while discriminative memory relies on other cortical areas, such as the perirhinal cortex (Aggleton and Brown, 2005; Eacott and Gaffan, 2005; Ross and Slotnick, 2008). Thus, the different roles the H1 receptor seems to have in spatial and recognition memory might rely on those distinct anatomic requirements, since the density of H1Rs is different in the cortex and the hippocampus.

Administration of Histamine into the Hippocampus and Memory Performance

The hippocampal formation is composed of three subregions: the Ammon's horn, the dentate gyrus, and the subiculum. The Ammon's horn is further divided into the subfields CA1–CA4. These subregions are connected to form a closed-loop circuit with the adjacent entorhinal cortex. Neurons from the layer II of the EC project to the dentate gyrus and the CA3 subfield, while neurons from layer III project directly to CA1. The projections from the EC are known as the perforant pathway. Neurons from the DG project to the CA3 subfield, through the mossy fiber pathway. CA3 neurons make synapse with neurons from the CA1 region through the Schaffer collateral pathway, the CA1 region neurons project to the layer V of the entorhinal cortex(Suzuki *et al*, 1994).

The superficial entorhinal layers receive input from other cortical areas and the olfactory bulb. The CA1 area is the main output of the hippocampus, which is directed mainly to the EC, which act as a hub to other cortical areas. The hippocampus can also project to subcortical areas, mainly through the subiculum. Among the subcortical input to the hippocampus are projections from the septum, amygdala, thalamic nuclei, diagonal band of Broca, the basal nucleus of Meynert, the tuberomamillary nucleus, the ventral tegmental area, the raphe nuclei, and the locus coeruleus. Finally, the connectivity of the hippocampus to other structures differs with respect to its dorsoventral axis (Burwell *et al*, 1995; Lavenex *et al*, 2006).

The hippocampus is central in spatial memory tasks (Wang *et al*, 2010), and the NMDA receptor

plays an important role in this type of learning (Pitkänen *et al*, 1995). The impairment in radial maze learning induced by the NMDA receptor antagonist MK-801 could be reversed by the direct administration of histamine in the hippocampus (Huang *et al*, 2003). Since the blockage of the H3 receptor in this structure had a similar effect and the reversal was hindered by the administration of an H1R antagonist (Huang *et al*, 2003), the observed improvement might be due to the increased synaptic availability of histamine and its binding to postsynaptic H1 receptors (Huang *et al*, 2003). The modulation of the excitability of hippocampal cells through the H1 and H2 receptors (Brown *et al*, 2001b) and the direct activation of the NMDA receptor through the polyamine site (Brown *et al*, 1996; Vorobjev *et al*, 2003) are possible mechanisms to mediate the observed effects.

The inhibition of the septum or the administration of scopolamine causes memory deficits that are reversed by histamine or the H3 receptor antagonist clobenpropit. Together with the mechanisms described for the modulation of the septohippocampal pathway (Xu *et al*, 2004), histamine seems to locally regulate ACh release in the hippocampus.

The hippocampus is also required for learning aversive tasks like inhibitory avoidance and contextual fear conditioning (Izquierdo *et al*, 1997). There is a possible anatomical dissociation within this region regarding the effects of histamine on aversive tasks, since in the dorsal hippocampus histamine seems to facilitate both fear conditioning and passive avoidance through the H2R, while in the ventral area, it has inhibitory effects on active avoidance through the H1R. The extent to which the methodological approaches of each aversive paradigm play a role in this dissociation remains unclear. In addition, there is evidence that the hippocampus has a functional segmentation in its dorso-ventral axis, with spatially distinct molecular domains and subdomains (Fanselow and Dong, 2010). This might lead to differences in receptor density or downstream signalling pathways that together could account for the observed results. Thus, the mechanisms that mediate the effects seen in this area may result from the interplay of histamine with cholinergic and other aminergic systems, direct activation of the glutamatergic system through the NMDA receptor, or by cellular downstream cascades activated from a particular receptor, with a possible overall combination of one or more mechanisms (Brown et al., 2001; Haas et al., 2008; Blandina et al) that altogether could account for discrepancies and differential involvement of histamine in a particular memory task.

Administration of Histamine into the Amygdala and Memory Performance

The amygdala is a complex mass of gray matter that comprises multiple and distinct subnuclei and is richly connected to nearby cortical areas that constitute the amygdala circuitry. The amygdala contains three functional subdivisions, each one having a unique set of connections with other regions of the brain. The medial group of subnuclei has extensive connections with the olfactory bulb and the olfactory cortex. The basolateral group, which is especially large in humans, has major connections with the cerebral cortex, especially the orbital and medial prefrontal cortex. The central and anterior group of nuclei is characterized by connections with the brainstem and hypothalamus and with visceral sensory structures, such as the nucleus of the solitary tract. For more details about the general organization of the amygdala anatomy circuitry (Bliss *et al*, 1993).

Previous studies have clearly identified the amygdala as a key brain structure for acquisition and storage of several memory types, first among them, fear memory. Classical fear conditioning is a powerful behavioural paradigm that has been widely studied in amygdala nuclei and mainly in the basolateral amygdala nucleus (BLA), which have been shown to participate in the learning and memory consolidation mechanism (LeDoux, 2014). These findings are consistently supported by a large number of studies using different experimental paradigms and measures of aversive memory (Blair *et al*, 2001; Maren *et al*, 2013). In addition, the amygdala also modulates fear-related learning in the other brain structures, such as the cortex and the hippocampus (McGaugh, 2004).

The studies that investigate the modulatory effects of histamine in the BLA are summarized in s. Electrophysiological studies in rat brain slices revealed that histamine can have bidirectional effects on excitatory synaptic transmission in BLA depending on the blockage of H3Rs: the excitatory postsynaptic potential was depressed in the presence of histamine alone but increased when the H3 receptor antagonist thioperamide was added to the preparation (Lovenberg *et al*, 1999). Behavioural models, however, are better suited to study such effects, since the slice preparation lacks afferent and efferent projections.

Evidence using the fear conditioning task with pharmacological agonists/antagonists showed that blockage of the H3Rs has inhibitory actions, while their activation improves the expression of fear memory. It has been shown that the modulation of ACh release in amygdala through the H3R participates in this modulation, since rats that received the infusion of H3 receptor antagonists in the BLA at similar concentrations to those that affected fear memory had a significant reduction in the spontaneous release of ACh (Passani *et al*, 2001). New approaches using transgenic models agree with the inhibitory role for histamine in fear memory, since the lack of H1- and H2Rs improves memory for this task, and mice with reduced levels of histamine due to lack of histamine

decarboxylase also have improved performance although the inactivation was not restricted to the BLA. Along with pharmacological data, this suggests that these inhibitory effects might also be mediated by the H1- and H2Rs.

Administration of histamine in the Prefrontal Cortex and memory

The medial prefrontal cortex (mPFC) is critically involved in numerous cognitive functions, including attention, inhibitory control, habit formation, working memory and long-term memory. Moreover, through its dense interconnectivity with subcortical regions (e.g., thalamus, striatum, amygdala and hippocampus), the mPFC is thought to exert top-down executive control over the processing of aversive and appetitive stimuli. Because the mPFC has been implicated in the processing of a wide range of cognitive and emotional stimuli, it is thought to function as a central hub in the brain circuitry mediating symptoms of psychiatric disorders (Riga *et al*, 2014). The local mPFC network consists mainly of excitatory pyramidal cells (80–90% of the total population) and inhibitory GABAergic interneurons (10–20% of the total population), both of which can be further subdivided into different cell types based on morphological, physiological and molecular properties (Ascoli *et al*, 2008; DeFelipe *et al*, 2013). Working memory is a complex brain process that refers to temporary storage of information (time scale of seconds to minutes) necessary for cognitive performance (Baddeley, 1992). The mPFC has been implicated in this process as it was found that reversible pharmacological inactivation of the PLC impaired working memory performance (Gilmartin and Helmstetter, 2010). The mPFC is thought to exert cognitive control over conditioned responding to aversive and rewarding stimuli by integrating information about experienced contexts and events (Euston *et al*, 2012). The fear-conditioning paradigm is a widely used animal model to study learning and memory function, as well as extinction of acquired fear memories (LeDoux, 2014; Maren *et al*, 2013). Habits are defined as behavioural patterns that are insensitive to changes in outcome value. Habitual behaviour is differentially regulated by mPFC subareas; whereas the PLC promotes flexibility, ILC activation inhibits flexibility and promotes behavioural rigidity (Killcross and Coutureau, 2003). Previous studies demonstrated that lesion and pharmacological inactivation of the ILC induce a switch from fixed to flexible responding (Coutureau and Killcross, 2003).

Histamine modulates the activity of prefrontal cortex, resulting in the modification of mnemonic processes, interacting with other systems: after 3 mg/kg oral administration of GSK189254 an H3R-antagonist, increased *c-Fos* immunoreactivity in prefrontal and somatosensory cortex was observed. Microdialysis studies demonstrated that GSK189254, increased the release of acetylcholine, noradrenaline, and dopamine in the anterior cingulate cortex and acetylcholine in

the dorsal hippocampus (Medhurst *et al*, 2007a). ABT239, another H3R-antagonist, when orally administrated increases histamine release in different brain areas but also acetylcholine in the prefrontal cortex (Munari *et al*, 2013). The histaminergic system appears to differently, depending on receptor subtype involved, modulate extinction via acting on the prefrontal cortex, as demonstrated by Fiorenza and co-workers (Fiorenza *et al*, 2012).

Area- and Time-dependent control of histamine in aversive memory

Aversive memories can follow different processing routes, engaging multiple independent circuits. Emotionally relevant experiences activate the histaminergic system(Valdés *et al*, 2010), and there is abundant evidences demonstrating that central histamine, mostly via H2Rs in the BLA (Cangioli *et al*, 2002; Garbarg *et al*, 1980), the dorsal hippocampus (Arrang *et al*, 1983; da Silva *et al*, 2006; Giovannini *et al*, 2003) or the nucleus basalis magnocellularis (Benetti and Izquierdo, 2013) modulates the consolidation of memory associated to aversive events.

Benetti and colleagues (Benetti *et al*, 2015) examined IA memory formation in rats temporarily depleted of histamine by i.c.v. injections of α-FMHis, an irreversible histidine decarboxylase inhibitor, that completely suppressed spontaneous and evoked histamine release from the tuberomamillary nucleus: this work provides evidence that intact histamine neurotransmission is required specifically for the establishment of long-term aversive memory, whereas short-term memory formation is independent of histamine neurotransmission. Interestingly, the restoration of memory by local infusion of histamine strongly depended on the timing and brain region: whereas in the BLA only immediate post-training infusion of histamine allowed long-term memory formation, in the CA1 reinstatement of histamine consolidates aversive memory even 6 h after training, confirming that the hippocampus is engaged in IA memory processing for a period longer than the amygdala (Calderazzo Filho *et al*, 1977; Katche *et al*, 2013).

The important finding in Benetti's study is that the histaminergic transmission in the BLA is crucial for the early phase of IA memory consolidation that occurred despite the blockade of histaminergic neurotransmission in the hippocampus. This is surprising, given the important contribution of hippocampal histamine receptors to IA consolidation (da Silva *et al*, 2006; Giovannini *et al*, 2003).

A prudent interpretation of these results is that following the local activation of the histaminergic system, the BLA takes over the functions of the hippocampus in the consolidation process and renders the hippocampus no longer crucial for long-term memory formation (Benetti *et al*, 2015).

Memory retrieval

Retrieval is the use of learned information, induced by sensory or internal cues. In simple modified reflex behaviour, it refers to the post-experience readout of the experience-induced change in behaviour and in its underlying synaptic efficacy (Kandel and Schwartz, 1982). In memories encoded and stored in more complex circuits, such as distributed memories in the mammalian brain, retrieval is posited to involve distinct processes, including selection, reactivation or reconstruction of the target representation, and assessment of the outcome (Dudai, 2002; Tulving and Patterson, 1968). These sequential and parallel processes can be completed within a fraction of a second (e.g., (Thorpe *et al*, 1996)). Retrieval is critical to understanding memory. In fact, once encoding is over, memory unretrieved, whether naturally or by experimental manipulations, is undetected, hence retrieval of the engram or part of it is an essential part of the proof that the specific engram exists (Ben-Yakov *et al*, 2015).

Despite its central importance in the study of memory and the abundance of data and models of retrieval in human experimental psychology, until fairly recently, retrieval in complex neural circuitry remained mostly an uncharted terrain in the neuroscience of memory. This was owing to a multitude of hindrances, including difficulties in teasing apart retrieval from encoding, limited knowledge on localization of specific candidate memory circuits in humans and animals, and lack of neurobiological methods with the proper spatiotemporal resolution that permits monitoring and manipulation of these circuits to observe, block, trigger or enhance retrieval. The development of novel paradigms, model systems, and new tools in molecular genetics, electrophysiology, optogenetics, in situ microscopy and functional imaging, have contributed markedly in recent years to our ability to investigate retrieval and understand part of its processes and mechanisms from the cellular to the behavioural level. In this work, we will review some of these developments. We will begin with selected studies of memory retrieval in the rodent brain and proceed to discuss aspects of retrieval of episodic memory in the human brain (Ben-Yakov *et al*, 2015).

Memory retrieval in rodent brain

The study of memory retrieval in the mammalian brain assumes that the process involves reactivation of patterns of neural activity associated with the original experience, although not necessarily identical with the activity patterns that represented the original experience. Retrieval is hence considered as a reconstructive rather than a replicative process. This activity is likely to be sparse and anatomically distributed, with different brain regions contributing to the quality and

strength of the recall. Although the human work focuses on a richer psychology and more complex neuroanatomy than accessible in the rodent, a common theme in both strands of research is a search for coherent patterns of activation correlated with retrieval and for correlations between retrieval and initial learning. The rodent work has been spurred recently by techniques that allow direct activation of distributed neural ensembles to test their functional involvement in memory. We will focus first on the role of hippocampal and cortical circuits in the retrieval of explicit memories in the mouse and rat (Ben-Yakov *et al*, 2015).

Patterned Activity during Retrieval

As discussed in the literature, the hippocampus plays a critical role in explicit forms of memory that in rodents has been investigated extensively in relation to spatial learning (Morris *et al*, 1982). A striking feature of the rodent hippocampus is the identification of place cells (O'Keefe and Dostrovsky, 1971), neurons that fire when the animals enter specific locations in their environment (Moser *et al*, 2015). This has led to the view that the hippocampus forms a cognitive map; it encodes a map of space that can be used to allow recognition of specific environments, guide movement through the environment, and identify specific goal areas within an environment (O'Keefe *et al*, 1971). At a circuit level, the hippocampus (and surrounding structures such as entorhinal and perirhinal cortex) could serve this function by integrating multimodal sensory information to form a unique map of each particular environment, object, or event. During retrieval, when a sufficient partial set of cues is provided, the entire map (memory) is recruited in a manner that likely involves the activation of multiple cortical regions coordinated by the hippocampus. A first question we can ask is how similar is the pattern of neural activity during two retrieval trials, or during retrieval and initial learning? (Ben-Yakov *et al*, 2015).

One means of assessing neural activity is through examination of a group of genes, the immediate early genes (IEGs), whose expression in neurons is responsive to activity (Farivar *et al*, 2004). The most commonly used IEGs for neural activity mapping are *c-Fos*, *arc*, and *zif268*. The expression of these genes is modulated by a variety of second messenger signalling pathways, but in excitatory neurons these all seem to be linked to neural activity (Sagar *et al*, 1988; Squire *et al*, 1991). The *cfos* gene has been used most extensively in the analysis of rodent behaviour and is responsive to burst activity of 30 or more action potentials at frequencies of 10 Hz or above (Sagar *et al*, 1988; Schoenenberger *et al*, 2009). It has been used in many behavioural studies examining different brain areas and generally indicates activity in areas consistent with the known electrophysiological responses to the behaviour. Although the use of IEG expression offers a simple assay for neural activity with cellular resolution, it lacks temporal resolution, the ability to assess low levels of

activity—for example, single action potentials, and the ability to determine activity patterns at more than one time point, which is a requirement for determining the stability of neural ensemble activity during learning and retrieval or across multiple retrieval trials (Ben-Yakov *et al*, 2015).

The problem of determining activity patterns at two different time points with IEGs was addressed with a method called compartmental fluorescent in situ hybridization (catFISH) (Guzowski *et al*, 1999). This approach takes advantage of the fact that genes that are being actively transcribed will have unspliced mRNA in the nucleus but relatively little processed transcript in the cytoplasm at short time points after induction. Cells that were active in the past, but are currently silent, will have mRNA in the cytoplasm but no nuclear transcript. The approach was used with the *arc* gene to test ensemble activity in the hippocampus in animals that explore an identical environment twice (A-A) compared with animals exploring two distinct environments (A-B). The study found greater ensemble reactivation in CA1 neurons (40%) when the two environments explored were identical (A-A group) versus when they were distinct (A-B group, 15% reactivation). This result is consistent with the idea that the hippocampus is encoding a representation of place that is reactivated when the information is retrieved. However, there are a number of caveats to this interpretation. First, the experiment is really just comparing the activity pattern of two sensory experiences rather than retrieval of a memory. Second, the technique only allows the comparison of ensemble activity at two closely spaced time points (30 min or less) and so does not indicate the stability of these ensembles over long time frames or in multiple retrieval trials. Finally, the link of IEGs to neural activity is crude and does not allow precise assessment of activity patterns or low levels of activity as can be achieved with electrophysiological recording (Ben-Yakov *et al*, 2015).

Classical hippocampal recording techniques cannot readily detect large ensembles of neurons stably over long periods of time, making it difficult to use for the type of experiment discussed above. An alternative approach is the use of genetically encoded reporters of calcium levels combined with optical imaging of the transient fluorescent signals produced on calcium binding (Akerboom *et al*, 2012). This approach offers a level of temporal resolution and action potential sensitivity that is intermediate between physiological recording and IEG expression, with the advantage that the neurons are precisely identified anatomically and can be stably imaged over long time periods. This approach was used to simultaneously record the activity of between 500 and 1000 CA1 hippocampal neurons over 45 days while the animals ran on a linear track in a constant spatial environment (Ziv *et al*, 2013). As seen in previous electrophysiological studies, on any given day, the neurons showed clear spatial firing fields with ~20% of neurons meeting the criteria for place cells. However, between any 2 days, the precise ensemble of neurons recruited in

each session showed a lower level of stability. At 5 days separation between recording sessions, there was a 25% overlap in the identity of place cells recorded in the two sessions. The overlap dropped to 15% at 30 days separation between recording sessions with only ~3% of cells active in all 10 recording sessions. So while on each day 20% of the CA1 neurons were active as place cells on the track, the majority of those cells were different from day to day in identical spatial environments. If the hippocampus is representing the environment through the activity of place cells, then why is there not greater stability of the ensemble of neurons activated when the animal is re-exposed to that environment? (Ben-Yakov *et al*, 2015).

There are several possible explanations for the apparent instability in hippocampal ensembles in the preceding experiment. One possibility is that there are subtle differences in the environment from day to day to which the animal is responding. Another possibility is that the task (running for a water reward) was not sufficiently salient to produce a stable representation. A previous study in mice using tetrode recordings showed that the stability of place cells over several days is modulated by the salience of the task the animals were required to perform in that environment (Kentros *et al*, 2004). Having non-food-deprived animals collect food pellets during the recording caused the place cells to be unstable from day to day while requiring the animals to attend by demanding that they navigate to a specific location to avoid an aversive light/noise cue produced the greatest temporal stability in place cell firing. Another possibility is that the CA1 neurons are encoding a component of time such that each successive day the ensemble varies to indicate that, although the environmental cues are identical, today's exploration is a different event in time from the previous days. This was suggested by electrophysiological recording of place cells over hours to days where it was found that many CA1 neurons changed their firing rate over time, consistent with the calcium imaging studies above, although the CA3 neurons showed greater session-independent stability in firing pattern (Mankin *et al*, 2012). Thus, the CA3 region of the hippocampus may hold an environment-specific map with the CA1 region adding information related to the specific time the environment is explored. Finally, it is possible that the critical spatial signal is contained in the small percentage of neurons that do show consistent firing between multiple recording sessions or in subtleties in the activity patterns that are below the threshold of this technique to resolve (Ben-Yakov *et al*, 2015).

Whatever the explanation is, this experiment raises an important question in understanding how memories are represented and retrieved. How consistent is the pattern of brain activity in response to two identical sensory inputs or two recall events? What is noise and what is signal in the pattern of neural activity that is observed? Certainly the brain's ability to consistently

recognize and learn about elements in the environment implies some coherent signal in the neural activity patterns induced by the same sensory stimulation, but the models and approaches to understanding this information will differ depending on whether the signal is a dominant or a minor component of the sensory-evoked activity (Ben-Yakov *et al*, 2015).

The examination of activity patterns during learning and retrieval or during two bouts of memory retrieval provide one means of identifying a neural signal associated with memory recall. As we have seen above, these experiments provide somewhat conflicting data regarding the degree of ensemble reactivation fidelity during different retrieval events. These experiments are also conceptually problematic in that they do not truly differentiate memory retrieval from sensory processing or encoding. When animals are placed in the same environment twice, they experience the same sensory cues and any consistency in the neural ensemble activity could reflect the processing of these cues rather than memory retrieval. Finally, even if the neural activity represents memory retrieval, the data are correlative and would require direct manipulation to test for functional relevance (Ben-Yakov *et al*, 2015).

Optogenetic Manipulation during Retrieval

One approach to directly test the function of distributed ensembles of active neurons in the mouse has recently been developed (Reijmers *et al*, 2007). The approach uses a transgenic mouse that allows the genetic modification of neurons based on their natural, environmentally evoked, activity patterns within an experimentally controlled time window. The IEGs, discussed above, are genes that are expressed in response to neural activity and previous studies have shown that their DNA promoter elements could confer this activity-dependent expression on linked reporters in transgenic mice (Smeyne *et al*, 1992). This was exploited to develop a binary genetic system in which neural activity at a given point in time could drive expression of any gene of interest (GOI) in the active neurons. In this system, the cfos promoter is used to drive expression of the tetracycline transactivator (tTA), a transcription factor that can be modulated by the antibiotic doxycycline (Dox), a derivative of tetracycline. The tTA can activate the expression of a second gene when it is linked to a tetracycline-responsive promoter element (TRE). In animals carrying two transgenes, cfos-tTA and TRE-GOI, the expression of tTA is directly linked to natural neural activity by the cfos promoter rising and falling as neurons become active. In the presence of Dox the transcriptional activity of the tTA is blocked, preventing downstream activation from the GOI. When Dox is removed, the GOI will now be expressed (via tTA driven transcription) in all neurons that are sufficiently active to drive the cfos-linked tTA. This allows the genetic modification of neurons active at specific points in time in response to specific environmental stimuli, for example, learning

or retrieval cues.

The approach was used in several recent publications to test the psychological relevance of distributed neural ensembles activated during learning in a contextual fear-conditioning task (see Fanselow and Wassum 2015). In contextual fear conditioning, animals receive footshocks in a particular context (the conditioning box) that contains multimodal sensory cues (generally distinct visual, tactile, and odor cues). Memory retrieval is assessed by the fear response the animals display when returned to the conditioning chamber. This is a model of explicit memory that is sensitive to hippocampal lesions and, reported by a number of groups, to show the temporal gradient in amnesia seen in many studies of human hippocampal patients with older (consolidated) context memories insensitive to hippocampal lesion (Anagnostaras *et al*, 1999; Squire and Bayley, 2007). In Liu et al. (Liu *et al*, 2001), *cfos*-based genetic tagging was used to introduce channelrhodopsin (ChR2) into dentate gyrus (DG) neurons that were activated during contextual fear learning. Mice were placed in one of two contexts A or B (conditioning boxes with different visual, tactile, and odor cues) and the neurons that were naturally activated by this environmental exploration were tagged with ChR2 to allow their subsequent direct activation with light. Both groups of mice were then fear conditioned to context A while on Dox, to prevent any further labeling of active ensembles. When mice in which DG neurons were tagged in context A were also fear conditioned in context A, the subsequent stimulation of the ChR2 expressing neurons produced a fear response in a neutral environment. Stimulation of those neurons that were genetically tagged with ChR2 while animals explored context B failed to produce fear in animals conditioned to fear context A. This suggests that the ensemble of neurons activated during the exploration of context A is capable of producing memory retrieval when directly stimulated. That is, the brain represents the conditioning box through the activity of the ensemble of neurons active in the DG during initial exploration (learning) and presumably through downstream neurons in other brain regions that are activated when this ensemble is directly stimulated via ChR2.

It is quite remarkable that the highly non-physiological stimulation that is produced with ChR2, which fires all neurons simultaneously and thus eliminates any temporal sequence effects or coordination with endogenous rhythms, can apparently produce a coherent internal representation of a complex environment. To further explore this idea, a complementary experiment was performed to determine whether the ensemble activity in the DG could serve as a conditioned stimulus (CS) in fear conditioning. In this case, *c-Fos*-activated neurons were genetically tagged with ChR2 while the animals explored context A, as in the previous study.

However, now the ChR2 labeled ensemble was activated in a neutral context and paired with footshocks. Thus, the artificial stimulation served as a CS that was paired with a footshock unconditioned stimulus (US). In this case, the animals developed a fear response to context A even though they never actually received the aversive shock US in that environment (Ramirez *et al*, 2013). Taken together, these results provide support for the notion that the activity of DG neural ensembles is sufficient to represent the context and serve as a cue for memory encoding and retrieval.

The artificial stimulation of small ensembles of neurons in the DG region of the hippocampus allows the retrieval of contextual memories presumably by activating more distributed ensembles of neurons in downstream hippocampal and cortical regions. Given the highly parallel nature of neural connectivity and processing, it is possible that although these ensembles may be sufficient for retrieval, they are not necessary as other pathways could compensate. The question of necessity was addressed in two recent studies using a light-gated proton pump from archaebacteria (ArchT) to hyperpolarize and silence *c-fos* tagged neural ensembles in experiments analogous to those described above with ChR2 (Denny *et al*, 2014; Tanaka *et al*, 2014). Both teams of investigators also used context fear conditioning and examined the requirement for ensembles in three different hippocampal regions, the DG, CA3, and CA1. Expression of ArchT was driven into neurons that were active during learning in context fear conditioning, and these ensembles were subsequently silenced in either the DG, CA3, or CA1 during memory retrieval. In each case, the silencing impaired the retrieval of the contextual fear memory. These results show that in each of the three major hippocampal subregions, the reactivation of the ensemble of neurons active during initial learning is necessary for the subsequent retrieval of the memory. This is consistent with the view that retrieval involves the reconstruction of patterns of brain activity produced during initial learning (Ben-Yakov *et al*, 2015).

Retrieval of Consolidated Memories

Another notable aspect of memory and its retrieval is the change in circuit structure of hippocampal-dependent memories over time, originally exemplified in amnesic patients such as H.M. (Squire and Dede, 2015). The observation that newly formed memories require the hippocampus for retrieval, but following weeks (in rodents) or months to years (in humans), the hippocampus seems to become dispensable for at least some forms of explicit memory, suggests that there is an anatomical alteration in the memory over time. What is the nature of this circuit-based consolidation of memory (“systems consolidation”) (Squire *et al*, 2015)? What is the anatomical locus of hippocampal-dependent new memories and hippocampal-independent older,

consolidated, memories? What is the role of the hippocampus and cortex in the encoding and retrieval of new and old memories? These questions will also resurface below in our discussion of episodic retrieval in humans. The current view of this process posits that during initial encoding and retrieval of an explicit memory, the hippocampus plays a critical role, for example, by encoding an index or map that helps recruit the appropriate cortical regions and specific neural ensembles for recall. Over time, these cortical ensembles may become independent of the hippocampus, possibly by strengthening their connectivity through post-learning replay of relevant activity patterns (Wilson and McNaughton, 1994), such that with the newly strengthened cortical connections the memory, or at least some processed versions of it, can be retrieved independently of the hippocampus.

The evidence in rodents for this view of consolidated memories comes from lesion and inactivation of specific brain regions and from studies of the neural activity recruited during retrieval of recent and remote memories. One prediction of the classic memory consolidation model is that, although the hippocampus is important in recent memory retrieval, cortical areas should instead be required for remote memory retrieval, or possibly recent and remote retrieval. This has been tested in the consolidation of three different explicit memory paradigms in rodents: spatial memory, contextual memory, and an olfactory-based social memory (Bontempi *et al*, 1999; Frankland and Bontempi, 2005; Frankland *et al*, 2004). In all three studies, hippocampal lesions impaired recent memory retrieval (1 day post-training) but spared remote retrieval (30 days post-training) as expected for hippocampus-dependent tasks involving consolidation. For contextual fear and spatial memories, inactivation of the anterior cingulate cortex produced an impairment in remote memory retrieval but did not affect recent memory, the opposite profile to what is observed with hippocampal inactivation or lesion (Anagnostaras *et al*, 1999; Kim and Fanselow, 1992). A similar result was seen with prefrontal inactivation in the spatial task, while in the olfactory-based social task, inactivation of the orbital frontal cortex impaired both recent and remote memory retrieval.

In addition to inactivation and lesions to probe the anatomical structure of recent and remote memories in rodents, these studies also examined the neural activation patterns during retrieval using IEG expression. In each behavioural paradigm, the activation of the hippocampus was greater with retrieval of recent as opposed to remote memories. Conversely, a wide variety of cortical areas showed increased activity during the retrieval of remote relative to recent memories. Although these studies support the view that remote memories require an increased cortical role in processing, they also raise some questions regarding the initial model of

hippocampal coordination of cortical activity during retrieval of recent memory. If, during recent memory retrieval, the hippocampus is coordinating and recruiting cortical activity, then why are these regions often not required for retrieval at this time point and why is there altered cortical activity over time. This suggests that there may be a more fundamental alteration in the circuitry and nature of these memories over time. This is evident at the behavioural level as studies in mice (and humans) have shown that remote memories lose some specificity (Furman *et al*, 2012; Wiltgen and Silva, 2007; Winocur *et al*, 2010).

One recent study in the mouse has examined the cortical representation of explicit memory using tagging of behaviourally active neural ensembles with ChR2 (Cowansage *et al*, 2014). In experiments similar to those described above for the DG, ChR2 expression was driven into neurons that were activated during context fear leaning. The investigators examined the retrosplenial cortex, a cortical output area of the hippocampus that is required for encoding and retrieval of both recent and remote contextual memory (Keene and Bucci, 2008a, b). They found that artificial stimulation of the learning-activated ensemble (via ChR2) produced a freezing response suggesting that it instantiated a recall event similar to what was seen in the hippocampal studies. This shows a contextual representation capable of producing retrieval forms in the cortex, in addition to the hippocampus, at the time of learning. More surprisingly, they found that the artificial stimulation of these retrosplenial cortex ensembles produced fear recall even when the hippocampus was pharmacologically silenced 1 day after training, a time point when the hippocampus is still required for natural retrieval of contextual memories. These results show not only that a cortical representation for context memory forms at the time of learning but the normal requirement for the hippocampus in recall can be bypassed by direct activation of this representation. This finding is consistent with the view of the hippocampus as a map or index that recruits the appropriate cortical circuits during memory retrieval but does not directly store a necessary component of the consolidated representation itself (Ben-Yakov *et al*, 2015).

The experiments using ChR2 to reactivate neural ensembles that were naturally activated during learning provide important information on how the brain stores and represents complex information about the external world. However, it may be incorrect to characterize these neurons as the “engram” or engram-containing neurons as has sometimes been suggested. The term engram refers to the physical changes in the brain that underlie memory (Lashley, 1950). If we take context conditioning as an example, the conditioning box is initially neutral and exposure produces exploratory behaviour. When paired with footshock, the same chamber now causes animals to express a fear response. The engram for this memory would be the sites within the

brain that lead the same sensory information (the cues in the box) to be processed in a way that produces fear rather than exploratory behaviour, for example, by routing the information to activate the amygdala. The stimulation of ChR2 ensembles at any point in the pathway for processing the sensory information of the context might be expected to represent, and therefore substitute for, the context, without actually being the critical site of plasticity required to produce the processing to fear circuits (the engram). For example, if the retina could be artificially stimulated in precisely the same manner as when an animal explored the context then it might be expected to produce a fear response in conditioned animals in the same way as the experiments discussed above but without carrying the "engram" for that memory (Ben-Yakov *et al*, 2015).

The study of memory in invertebrates sets up a solid conceptual framework for understanding information processing by nervous systems. Sensory input is processed to different motor/behavioural output based on experience and via plastic changes at specific nodes in the processing network. The mammalian brain introduces a 4 (mouse)- to 7 (human)-order of magnitude increase in the number of neurons between the input and output nodes, but a deep understanding of mammalian memory and retrieval will still require identifying how this information flows through and is represented in the nervous system and the specific sites that are altered with experience to produce the retrievable memory. We have introduced some of the techniques used in mice and rat models and results that suggest a framework for probing this processing at a fine level focused on specific neural ensembles. The added complexity of the mammalian brain has allowed greater specialization of regions for different processing tasks, and memory/retrieval will likely involve a coordinated interplay of more sparse and distributed networks than in simpler systems. The work in humans involving functional brain imaging and, more recently, electrophysiology, is also beginning to identify some of the principles of these processing networks. These techniques allow the assessment of activity across the entire brain during encoding and retrieval and provide a view of the interactions and functional distinctions of different areas during processing (Ben-Yakov *et al*, 2015).

Memory retrieval in the human brain

Our discussion of human memory retrieval will focus on declarative and particularly episodic memory (Squire *et al*, 2015). We will start by briefly reviewing aspects of the rich phenomenological analyses of human memory retrieval, which has laid the foundations for much of contemporary research on brain substrates and processes of retrieval. We will then survey current knowledge on human brain circuits that sub-serve retrieval, and conclude with a brief description of functional models that inform many brain studies of long-term memory retrieval.

Phenomenological Analyses of Human Retrieval Processes

Contemporary research in the neurobiology of human memory retrieval relies heavily on a rich body of research in experimental and cognitive psychology that flourished already half a century ago. This research yielded classifications, models, and questions that are at the forefront of investigation of brain and behavioural mechanisms of retrieval.

A major distinction in human declarative retrieval is between recall and recognition. Whereas recall is the reactivation or reconstruction of the internal representation of a target item in the absence of that item, prompted by implicit or explicit cues, recognition is the judgment of previous occurrence in the presence of at least part of the target item. This distinction refers hence both to the test used to probe the memory (i.e., whether in the presence or in the absence of the target item) and to the postulated cognitive underpinning of the memory performance gauged by the test. Recall and recognition were each proposed to involve multiple processes, and these processes themselves were further dissociated. In brief, recall was initially posited by some to consist of two major phases, a “generation phase” followed by a recognition phase (Bahrick, 1970). Similarly, “dual-process” models of recognition maintain that recognition judgments can be based on two distinct types of memory, familiarity and recollection (Jacoby and Dallas, 1981; Voss and Paller, 2010; Yonelinas and Jacoby, 1994). Multiple experimental paradigms have been used to dissociate the two processes (Yonelinas and Levy, 2002), probing either a subjective sense of recollection (participants indicate whether they recollect an item, or find it familiar in the absence of specific recollection(Tulving, 1987)), or the objective ability to recollect additional aspects of the study event, such as the context in which it was learned (“source memory”) or an item associated with the probe during learning.

Although many investigators agree on the existence of two distinct recognition processes (but see (Shimamura and Kajimoto, 2010), for a single-process model), there remain several contentious debates. The first of these pertains to the nature of the recollection signal and how recollection

and familiarity ultimately contribute to the recognition judgment. The two leading classes of models, each based on both behavioural and functional neuroimaging evidence, differ primarily with respect to their view of recollection as either a threshold process or as a continuous variable. According to the dual-process, signal-detection model (Yonelinas *et al*, 1994; Yonelinas *et al*, 2002; Yonelinas and Parks, 2007), familiarity and recollection are two independent processes initiated in parallel. Familiarity is considered to reflect a continuous measure of memory strength, best modelled as a signal-detection process. Conversely, recollection is considered to be a threshold process, whereby only items falling above a certain threshold will be recollected, resulting in relatively high-confidence responses (Yonelinas *et al*, 2011).

According to an alternate approach, recollection is also a continuous measure that is best modelled by a signal-detection process, and recognition judgments are based on an aggregated memory-strength variable (Mickes *et al*, 2009; Rotello *et al*, 2004; Wixted, 2007). The continuous dual-process model (Wixted *et al*, 2010) maintains that, during the process of recognition, recollection and familiarity each elicit a separate internal measure along a memory strength axis. The decision criterion for identifying an item as recognized may be based either on one of these axes, or on an aggregated memory-strength axis, which takes into account both the sense of recollection and the sense of familiarity, depending on the task at hand. The different approaches start to converge, with suggestions that recollection may be graded based on the amount of recollected information (Rugg and Vilberg, 2013), but still subject to a threshold-like process (Yonelinas *et al*, 2011).

Another point of dispute is whether the tests typically used to dissociate recollection and familiarity (e.g., remember/know test 5) indeed dissociate these two processes (e.g.,(Yonelinas *et al*, 2002)), or whether they in fact only separate strong memories from weaker ones (Schonfield and Donaldson, 1966). Last, but not least, in the context of the present discussion, the debates involve the role of distinct brain regions, primarily in the medial temporal lobe (MTL) in recollection/familiarity processes.

We selected as an example the recollection/familiarity debate as it is currently one of the more heated topics in research on episodic memory retrieval (Voss *et al*, 2010). However, it is noteworthy that this far from covers the research into the phenomenology, and ultimately candidate brain mechanisms, of human memory retrieval. An additional distinction is drawn between the content of retrieval and the entering of a state that enables retrieval ("retrieval mode" (Tulving, 1987), see (Rugg and Wilding, 2000), for a more fine-grained fractionation), or more generally between different types of item-specific and item-invariant processes in retrieval

(e.g., (Buckner *et al*, 1998; Dobbins and Wagner, 2005; Nyberg *et al*, 1995)). Further, different types of memories have been shown to involve different retrieval processes, for example, when comparing autobiographical and laboratory-based memories (Svoboda *et al*, 2006), field versus observer perspective (Eich *et al*, 2009), objective versus subjective measures of recollection and emotional versus neutral memories (LaBar and Cabeza, 2006; Maratos and Rugg, 2001). Although this limited discussion does not allow for a comprehensive review of the fine-grained analysis of the phenomenology of human retrieval, it is important to bear in mind that the rich differentiation of retrieval process has been shown to manifest in differential brain activity during retrieval, emphasizing the notion that retrieval cannot be investigated as a unitary construct.

Substrates of Retrieval in the Human Brain

As found in rodents, brain circuits of human explicit retrieval are highly distributed (Maguire *et al*, 2000; Mendelsohn *et al*, 2010; Svoboda *et al*, 2006).

Hippocampus and MTL

Much of the emphasis in the study of episodic retrieval has been placed on the roles of the MTL (Squire *et al*, 2015), specifically differentiating between the hippocampus and surrounding cortices. As focal damage to the fornix and mammillary bodies results in impaired performance that resembles that of hippocampal damage (Rudebeck *et al*, 2009; Tsivilis *et al*, 2008), it has been suggested the study of hippocampus-based memory should be extended to include the fornix and mammillary bodies as part of the "extended hippocampal system" (Aggleton and Brown, 1999).

One approach to differentiating hippocampal versus MTL cortical contributions to retrieval is based on the aforementioned recollection/familiarity distinction. Several studies have addressed the question of whether the hippocampus is uniquely involved in recollection, or whether it supports both familiarity and recollection (Brown and Aggleton, 2001a; Eichenbaum *et al*, 2007; Rugg *et al*, 2013; Wixted *et al*, 2010). Both viewpoints are based on findings from studies in patients with MTL damage combined with functional neuroimaging results in healthy subjects.

Whereas many studies report a disproportionate effect of hippocampal damage on recollection and associative memory relative to familiarity (Aggleton *et al*, 2005; Giovanello *et al*, 2003; Huppert, 1981; Vargha-Khadem *et al*, 1997; Yonelinas *et al*, 2002), other reports find that hippocampal damage impacts familiarity and recollection to a similar extent (Cipolotti and Bird, 2006; Jeneson *et al*, 2010; Manns and Squire, 1999; Song *et al*, 2011). Interestingly, a patient with significant perirhinal damage that spared the hippocampus showed impaired familiarity and preserved recollection (Bowles *et al*, 2007). Such studies indicate a causal role of MTL regions in

the different processes, yet do not allow for dissociation between encoding, storage and retrieval, nor do they necessarily reflect memory processes in the healthy brain.

Conversely, functional magnetic resonance imaging (fMRI) studies do not allow for demonstration of causality, but they enable targeted investigation of correlation with different stages of memory. However, even in fMRI studies focusing on retrieval processes, incidental encoding during retrieval tasks may hinder the ability to tease apart encoding and retrieval processes ((Stark and Okado, 2003) but see Ben-Yakov (Ben-Yakov *et al*, 2014) for temporal dissociations between encoding and retrieval)). As with the patient studies, fMRI studies have led to divergent results regarding hippocampal involvement in recollection. One set of studies finds that the hippocampus subserves recollection, but not familiarity, of memoranda such as words or pictures (Diana *et al*, 2010; Eldridge *et al*, 2005; Montaldi *et al*, 2006; Rugg *et al*, 2013; Wheeler and Buckner, 2004; Yonelinas *et al*, 2002). More specifically, the hippocampus has been found to respond more strongly to words or pictures reported as "remembered" (Eldridge *et al*, 2005; Montaldi *et al*, 2006; Wheeler *et al*, 2004), in correct versus incorrect retrieval of the encoding context (Weis *et al*, 2004), and in associative relative to nonassociative recognition (Jeneson *et al*, 2010) or recognition of compound words (Ford *et al*, 2010). Recent studies propose that hippocampal activity is not related to the subjective sense of recollection, but modulated by the amount of contextual information actually retrieved (Rugg *et al*, 2013).

According to an alternate view, the hippocampus is not preferentially involved in recollection versus familiarity when controlling for memory strength at the time of the retrieval test (Montaldi *et al*, 2006; Wais *et al*, 2010a; Wais *et al*, 2010b). Wixted and colleagues suggest that the hippocampus and perirhinal cortex are involved in both familiarity and recollection (Squire, 2004), but that the hippocampus supports strong memories, whereas the perirhinal supports weak memories . However, they propose that memory strength is itself not the parameter that differentiates the hippocampus from the surrounding structures, but rather that different MTL structures process attributes of the memory that are differentially expressed in strong versus weak memories. According to this view, the hippocampus supports both recollection-based and familiarity-based recognition of multiattribute stimuli, and its involvement in retrieval is most evident for strong memories (Wixted *et al*, 2010). Despite the differing interpretations with respect to recollection/familiarity, overall, the hippocampus appears to be primarily involved in the retrieval of strong, rich, multi-attribute memories, whereas the surrounding cortices can support retrieval of more simple memories without hippocampal involvement.

Recollection/familiarity are often used in the human literature to describe both the behavioral

phenomena and the patterns of brain activity correlated with these phenomena, although there may not necessarily exist a one-to-one mapping between the two types of measures (Voss *et al*, 2010). A key example is the finding that the perirhinal cortex is involved in associative memory recognition under conditions of unitization, in which the associated elements comprise a unitized item (Diana *et al*, 2010; Ford *et al*, 2010), whereas the hippocampus is preferentially involved in recognition of non-unitized versus unitized pairs (Ford *et al*, 2010). This has been interpreted either as evidence that familiarity can support associative recognition when the paired associates can be bound into a compound unit (Ford *et al*, 2010) or as evidence that the perirhinal cortex is involved in recollection of unitized pairs (Diana *et al*, 2010). Similarly, findings of hippocampal neurons sensitive to picture novelty/oldness (Rutishauser *et al*, 2008; Viskontas *et al*, 2006) have been subject to differing interpretations, as it is not clear how neuronal sensitivity to familiarity relates to familiarity at the behavioural level (Wixted, 2007; Yonelinas *et al*, 2007).

The attempts to delineate the role of hippocampus in recollection versus familiarity rest on the assumption that the hippocampus honours the well-established behavioral dissociation between these two manifestations of retrieval. However, there is an increasing view that the behavioral distinction between recollection and familiarity does not reflect the underlying basic computational role(s) of the hippocampus and its surrounding cortices (Diana *et al*, 2010; Voss *et al*, 2010; Wixted *et al*, 2010). An alternate approach to interpreting the findings reviewed above is that the hippocampal formation plays a role in the binding items in their context (Diana *et al*, 2007; Eichenbaum *et al*, 2007). This postulated role also introduces a functional distinction between perirhinal and parahippocampal cortices, according to which the perirhinal cortex encodes item information, the parahippocampal cortex encodes contextual information, and the hippocampus encodes item–context associations as well as item–item associations. A related view (Montaldi *et al*, 2006) assigns to the perirhinal cortex a role in item–memory and within-domain inter-item associations. All of these models emphasize the role of the hippocampus in retrieval of bound associations relative to single item/context recognition, which are supported by the surrounding cortices. They can also be considered in line with the view, mentioned above in discussing the rodent work, that the hippocampus serves as a map or index that recruits the appropriate cortical circuits.

The aforementioned models predict a role for perirhinal cortex in recognition of single items. This is supported by fMRI studies that find decreased perirhinal activity for highly familiar stimuli (Montaldi *et al*, 2006; Weis *et al*, 2004), as well as an intracranial recording study that found reduced firing in response to familiar images in perirhinal cortex (Viskontas *et al*, 2006). A study

combining iEEG in patients with fMRI in healthy controls (Staresina *et al*, 2006) paints a more complex picture. In this study, the perirhinal cortex showed both an early-item novelty effect (differential response to novel vs. familiar words, potentially reflecting a familiarity process) and a sustained source retrieval effect (differential response to correct vs. incorrect retrieval of the context associated with the word). The hippocampus showed an early source retrieval effect, followed by a late-item novelty effect (potentially underlying encoding of the novel item). These studies show a clear role of perirhinal cortex in the recognition of familiar stimuli.

All in all, these different views converge on a predominant role of the hippocampus in associative retrieval. In addition to the functional division between the hippocampus and surrounding cortices, a within-hippocampus functional dissociation has also been suggested, with anterior hippocampus more involved in encoding and posterior hippocampus more involved in retrieval (Lepage *et al*, 1998; Poppenk *et al*, 2013).

Systems modulating memory recall

Stress exposure or the glucocorticoid corticosterone administered systemically shortly before testing for memory of inhibitory avoidance, contextual fear conditioning or water-maze spatial tasks (24 hours earlier) produces temporary impairment of retention performance (Cai *et al*, 2006; de Quervain *et al*, 1998; Sajadi *et al*, 2006). As the same treatments administered shortly before training do not affect either acquisition or retention performance assessed immediately after acquisition, such findings indicate that glucocorticoids impair retention by influencing memory retrieval. These findings are consistent with those indicating that stress exposure or glucocorticoids administered immediately after a learning session also impair retention performance tested 30–60 minutes after the session, i.e. at a time when the memory trace has not yet been consolidated into long-term memory (Diamond *et al*, 1999). Similarly, as is found with memory consolidation, glucocorticoid effects on memory retrieval require concurrent activation of noradrenergic mechanisms. The β-adrenoceptor antagonist propranolol administered systemically 30 minutes before inhibitory avoidance retention testing blocks the memory retrieval impairment induced by concurrent injections of corticosterone (Roozendaal *et al*, 2004b). The finding that stimulation of β1-adrenoceptors with systemic injections of the selective agonist xamoterol induces memory retrieval impairment comparable to that seen after corticosterone administration (Roozendaal *et al*, 2004a), suggests that glucocorticoid effects on memory retrieval impairment involve activation of noradrenergic mechanisms.

Peripheral administration of the opioid peptidergic antagonist naloxone or D2 dopamine receptor antagonists also blocks the impairing effect of concurrently administered corticosterone or dexamethasone on memory retrieval (Rashidy-Pour *et al*, 2004). Memory retrieval is also influenced by systemic administration of drugs affecting several other modulatory systems, including epinephrine, adrenocorticotropin, β -endorphin, vasopressin, acetylcholine and serotonin (Izquierdo *et al*, 2002). In investigating drug effects on learning and memory, including memory retrieval, it is critically important to distinguish the effects of the drugs on memory retrieval from those on other processes that may affect the behaviour used to assess memory.

Many studies have reported evidence that the hippocampus is involved in retrieval of spatial and contextual information (Kandel *et al*, 2000). Inactivation of the hippocampus with local infusions of the glutamatergic AMPA/kainate receptor antagonist LY326325 or the GABAergic agonist muscimol (45 minutes before testing) impairs memory retrieval of water-maze spatial and contextual fear conditioning tasks (Holt and Maren, 1999). As the GR agonist RU 28362 administered into the hippocampus shortly before retention testing also impairs retrieval of spatial memory (Roozendaal *et al*, 2004a), such findings indicate that glucocorticoid-induced memory retrieval impairment depends, in part, on GR activation in the hippocampus. Other studies have shown that the effect of novelty stress on memory retrieval is blocked by intrahippocampal infusions of the AMPA receptor antagonist CNQX, the metabotropic glutamate receptor antagonist MCPG, as well as the cAMP blocker Rp-cAMPs (Izquierdo *et al*, 2000). In contrast, infusions of the protein-synthesis inhibitor anisomycin do not block corticosterone effects on memory retrieval (Sajadi *et al*, 2006), suggesting that stress and corticosterone may influence memory retrieval through a protein synthesis-independent mechanism, a finding consistent with the rapid onset of stress and glucocorticoid effects on memory retrieval. Retrieval of memory of emotionally arousing information also induces activation of the BLA (Boujabit, Bontempi, Destrade & Gisquet-Verrier, 2003; Hall, Thomas & Everitt, 2001). Furthermore, intra-BLA infusions of norepinephrine or CNQX affect retrieval of memory for inhibitory avoidance training (Barros et al., 2001; Liang et al., 1996). In contrast, intra-BLA infusions of a GR agonist do not appear to affect memory retrieval (Roozendaal, 2003). However, the BLA interacts with the hippocampus in mediating glucocorticoid effects on memory retrieval. Lesions of the BLA or infusions of a β-adrenoceptor antagonist into the BLA block the impairing effect of a GR agonist infused into the hippocampus before memory retrieval (Roozendaal *et al*, 2004b). The findings of studies examining stress hormone effects on memory retrieval in humans are consistent with those of animal experiments and indicate that glucocorticoids impair memory retrieval via an

interaction with noradrenergic mechanisms. Stress-level cortisol or cortisone administration to human subjects impairs memory retrieval of emotionally arousing information or during emotionally arousing test conditions.

Imaging studies indicate that glucocorticoid effects on memory retrieval in human subjects are also mediated, at least in part, by actions in the hippocampus (de Quervain *et al*, 1998). However, other results of human imaging studies indicate that the amygdala is also activated during the retrieval of previously learned emotionally arousing material and indicate that the effect is independent of the valence of the emotional material (Dolan *et al*, 2000). Further, findings of human brain imaging studies are consistent with findings of animal studies indicating that the amygdala and hippocampus interact during the retrieval of emotionally arousing information (Smith *et al*, 2006).

Memory recall and intracellular pathways: *c-Fos* and CREB

Memory is represented by a sparsely distributed, specific collection of neurons in the brain that forms a unique memory trace (Mendez and Fras, 2011). Accordingly, only a portion of eligible neurons are recruited into a specific memory. During fear conditioning, about 70% of neurons in the amygdala, a critical brain site for fear memory acquisition and storage (LeDoux, 2014), are thought to receive sensory inputs, but only a percentage exhibits learning-related synaptic plasticity (Rumpel *et al*, 2005). The induction of IEGs is rapid and transient and of these genes, one of the most studied is *c-Fos* as it is considered a marker of neuronal activation (Herrera and Robertson, 1996). A study of the amygdala circuitry that combined the genetic tagging of *c-Fos–active* neurons and immediate-early gene imaging found that neurons in the basolateral amygdala that are activated during fear conditioning are reactivated during fear memory recall, but only small portions of neurons activated during training are reactivated (Reijmers *et al*, 2007). Liu and colleagues in 2012 demonstrated the relation between the activation of specific subpopulations of hippocampal neurons, positive for *c-Fos*, and the efficacy of recall of contextual fear conditioning engram in rodents; because recall can activate different pathways compared to those needed for acquisition and consolidation, even the IEG *c-Fos* seems to be differently expressed, in terms of time (Bontempi *et al*, 1999). The cAMP response element binding protein (CREB) is a nuclear factor that is regulated by protein kinase A phosphorylation. Transcription is stimulated on binding to the CRE of a phosphorylated CREB dimer (Gonzalez and Avendano, 1989). In 1990s several landmark studies defined CREB as “the memory gene” (Bourtchuladze *et al*, 1994). CREB depicts the convergence of different activity-driven kinase pathways, modulates different signalling cascades,

such as the increase of intracellular cAMP after activation of G protein-coupled receptors, the increase of Ca^{2+} through activation of voltage- or ligand-gated channels, or the activation of receptor tyrosine kinases by growth factors (Lonze and Ginty, 2002). CREB phosphorylation is upstreams of the so-called "plasticity-related proteins" (PRPs), such as neurotransmitters receptors, structural proteins, and adhesion and signalling molecule, BDNF, all regulating synaptic activity (Lonze *et al*, 2002). Accordingly, the activation of CREB due to synaptic activity elicited by a behavioural experience would induce the expression of molecules that are necessary for the stabilization of the structural and functional changes of synaptic strength encoding and expressing the memory engram for that experience (Benito and Barco, 2010). Han and colleagues found that neurons expressing elevated levels of cAMP response-element binding protein (CREB neurons) in lateral amygdala at the time of fear learning are preferentially selected for inclusion into fear memory trace and are essential for the later expression of that memory. As such, these neurons may represent key components in the fear memory trace (Cai *et al*, 2006). Current views on the dynamic nature of the memory trace (Dudai, 2002) suggest that recall is not just a simple neural process of memory expression, but can induce the processes of modifying recalled memory. In addition, it is believed that subpopulations of neurons that are active during behaviour training may be reactivated later in the brain and that this activity replay or reactivations of a memory trace may contribute to strengthening of a previously acquired memory (Inda *et al*, 2011; Sara, 2000). Liu and coworkers (2012) with an optogenetic technique reported that the reactivation of the dentate gyrus of the hippocampus that is active during contextual fear memory training is sufficient to drive the recall of that memory and in other set of experiments the same group described how the artificial activation of a subset of lateral amygdala neurons expressing elevated levels of CREB at the time of fear conditioning is sufficient for the recall of the fear memory (Akerboom *et al*, 2012).

Fear memory recall, necessary and dangerous: interventions in exposure-based therapy of fear, anxiety and trauma-related disorders

Fear memory in the most studied type of memory. It is easily and quickly learned and retained for a long time or even for a lifetime: it is a motivational system essential for organisms survival having a central role in organization of defensive behaviours to threat (Baldi and Bucherelli, 2015). When the reaction to fearful memories is excessive and disproportionate to the stimulus it becomes pathological.

Fear, anxiety and trauma-related disorders are associated with excessive fear reactions triggered by specific objects, situations or internal and external cues in the absence of any actual danger, and it is often characterized by an inability to extinguish learned fear and to show adequate safety learning (Jovanovic *et al*, 2013; Milad *et al*, 2013). Pathological fear and anxiety are present in a variety of psychiatric disorders, including types of phobia, panic disorders, obsessive-compulsive disorder, generalized anxiety and post-traumatic stress (DSM-5, 2013; ICD-10, 1994). These disorders comprehend the most widespread mental illness and are estimated to have a lifetime prevalence of up to 28% among western population (Wittchen *et al*, 2011) and the personal suffering of patients, the economic burden is heavy (Gustavsson *et al*, 2011).

To date pharmacological and psychotherapeutic treatments (Hasan *et al*, 2012) whose aim is to reduce fear and anxiety are associated with decreased severity but only a percentage of patients show long-term benefits as the majority fail to reach the complete remission (Hasan *et al*, 2013). With these considerations in mind, it is necessary to find alternatives. Pharmacological approaches either induce rapid anxiolytic effects (e.g. benzodiazepines, some antipsychotics) or require prolonged, chronic treatment (e.g. antidepressants) and all of these are symptomatic. Commonly used psychotherapeutic interventions apply cognitive behavioural strategies and exposure techniques to help patients overcome the maladaptive beliefs and avoidance behaviours that reinforce the pathology related to fear-eliciting cues. Meta-analyses show that cognitive behavioural therapy (CBT) does have efficacy for several anxiety disorders, including post-traumatic stress disorder, but patients have difficulty bearing the demanding and exhausting process of therapy and many who do manage to cope with it respond only partially and often relapse with time (Choy *et al*, 2007).

Enhancing fear extinction to treat fear–related disorders

The extinction of fear conditioning refers to the decrease in fear responses during repeated presentations of the conditioned stimulus without unconditioned stimulus reinforcement. Extinction can refer to the within-session decrement in fear responses while animals are receiving presentations of the conditioned stimulus alone during extinction training. It can also refer to the retention of extinction learning when animals are presented with the conditioned stimulus at later time points. Extinction is thought to involve new learning rather than erasure or unlearning of the association. Evidence for this assertion comes from the observation that fear responses spontaneously recover with passage of time (Quirk, 2002), that fear responses show renewed responding when the conditioned stimulus is presented in a different environmental context from that in which extinction training occurred (Bouton and King, 1983), and that presentation of the unconditioned stimulus alone reinstates fear to a cue that has undergone extinction training (Rescorla and Heth, 1975). The extinction of fear conditioning relies on some of the same brain circuitry necessary for acquiring fear memories, including the amygdala and hippocampus (Corcoran *et al*, 2005). There is good evidence that extinction also requires activity of the vmPFC, which is not normally involved in the acquisition of fear conditioning. In rats, the infralimbic portion of the vmPFC appears to be critical for the extinction of fear conditioning. Lesions of this area have been shown to disrupt the retention of extinction (Quirk, 2002), and neurons in the infralimbic cortex show increased firing during the recall of extinction memory (Milad and Quirk, 2002). Neurons in the infralimbic cortex are thought to decrease fear responses by means of projections to GABAergic intercalated neurons positioned between the lateral or basal and the central nuclei of the amygdala, which inhibit the output of the central nucleus.

Studies of extinction learning in humans largely parallel studies in rats, demonstrating that the vmPFC (Carrion *et al*, 2010), amygdala and hippocampus (LaBar *et al*, 2006) are all engaged during extinction learning or the recall of extinction. Pharmacological approaches that enhance fear extinction are being evaluated for treatment efficacy in PTSD. The use of D-cycloserine (DCS), a partial NMDA receptor agonist, as a potential treatment for PTSD arose as a result of many preclinical studies implicating NMDA receptor activity in learning and memory processes (Reingle *et al*, 2014). DCS was first tried in humans for anxiety disorders in combination with virtual reality exposure (VRE) therapy for the fear of heights (Ressler *et al*, 2004). After treatment, those patients that received DCS in combination with VRE showed greater improvement than those who received placebo and VRE. Since that study, DCS has been shown to be an effective therapeutic compound for increasing the rate of recovery with exposure-based psychotherapy several fear-

and anxiety-related disorders, including panic disorder, social anxiety disorder, obsessive-compulsive disorder and PTSD (de Kleine *et al*, 2012) . Although there have been some negative trials, most of these can be explained retrospectively as the mechanism of DCS is further understood, and two recent meta-analyses support the conclusion that it is an effective strategy to enhance the rate of emotional learning underlying exposure-based psychotherapy (Bontempo *et al*, 2012). Other methods of augmenting NMDA receptor activity in conjunction with extinction are also now being explored.

More recent work has identified brain-derived neurotrophic factor (BDNF) as a molecular target for facilitating extinction learning and a potential treatment for fear disorders (Andero and Ressler, 2012). Studies have shown that blocking the activity of BDNF in the amygdala (Chhatwal *et al*, 2006) or hippocampus (Heldt *et al*, 2007) disrupts the retention of extinction. Other studies indicate that memory for extinction can be facilitated by infusion of recombinant BDNF in the infralimbic cortex or dorsal hippocampus or by systemic injection of an agonist of its receptor TrkB (Andero *et al*, 2012). Further work involves the Val66Met variant of BDNF in humans. Carriers of the methionine-encoding allele release less BDNF peptide (Duggan *et al*, 2003) and have diminished extinction of conditioned fear (Soliman *et al*, 2010), which may explain the increased prevalence of anxiety-related disorders in people with this genotype (Rakofsky *et al*, 2012). Most intriguingly, in the same study (Soliman *et al*, 2010), it was shown in 'humanized' mouse models using knock-ins of each of the human alleles to the mouse Bdnf gene locus that these alleles lead to phenotypes in mice similar to those in human: decreased extinction of fear in the methionine allele carriers relative to that in the valine allele carriers. Some meta-analyses have failed to find increased incidence of anxiety disorders in methionine allele carriers (Frustaci *et al*, 2008); however, this might be the result of low samples sizes. Together these data extend our understanding and appreciation of the role of BDNF in extinction and recovery from fear and fear-related disorders. They also provide further evidence for the face validity of the usefulness of the extinction-of-fear model in mice for extinction of fear in humans.

Disrupting traumatic memories after retrieval

Recently there has been renewed interest in the notion that LTM becomes susceptible to disruption after a consolidated memory is retrieved. In fear conditioning studies, memory is retrieved by presenting the animal with a single presentation of the conditioned stimulus used to signal shock during acquisition. The seminal finding was that when a protein synthesis inhibitor is given after retrieval, LTM is impaired on subsequent tests (Nader *et al*, 2000). This result generated wide interest, and this phenomenon, termed reconsolidation, has now been observed

in organisms ranging from invertebrates to humans (Nader and Hardt, 2009). Less is known about memory reconsolidation than about initial consolidation, but the available evidence suggests that the molecular and cellular mechanisms supporting reconsolidation are similar to those necessary for consolidation, although they do not overlap completely (Tronson and Taylor, 2007).

The observation that fear memories can be disrupted by combining retrieval of memory with drug treatment open the possibility of using this strategy to treat fear-related disorders. Theoretically, patients could be brought into a clinical setting, presented with a stimulus that retrieves the fearful memory and given a drug, and the fear memory would be weakened. Recent laboratory studies have used this basic approach to determine whether fear memories can be disrupted by combining retrieval with a memory-impairing drug. In one study (Kindt *et al*, 2009), human subjects were fear conditioned, given a retrieval trial the next day in conjunction with oral administration of the β-adrenergic blocker propranolol, and tested the day after. The results showed that those given the drug while the memory was reactivated showed significantly less fear-potentiated startle during testing the next day than those given placebo. At least one study (Brunet *et al*, 2008) has shown that a similar approach can be taken to disrupt traumatic memories in humans. In this study, PTSD patients were asked to describe a traumatic experience and were given a single dose of propranolol or a placebo. Patients given propranolol showed reduced physiological signs of fear when they were asked to once again describe the traumatic experience a week later.

Although there are some differences, there is also evidence that disruption of reconsolidation and extinction may share some properties (Fiorenza *et al*, 2012). Of note, in vivo and ex vivo physiological studies have suggested that fear learning leads to LTP-like potentiation of synapses with fear learning. Extinction of fear then appears to be associated with depotentiation and LTD-like mechanisms in some models (Mao *et al*, 2006). Thus, diminished representation of synaptic strength may be achieved, in part, both through strengthened extinction and through inhibited reconsolidation. Although this strategy is promising, laboratory studies of reconsolidation indicate that there may be limitations to using a reconsolidation-disruption approach as a way to treat fear-related disorders. Several studies have indicated that retrieval does not always trigger reconsolidation, including the observation that both older and stronger memories are less susceptible to disruption after retrieval (Mao *et al*, 2006). If this pattern of data extends to humans with fear-related disorders, it may prove difficult to disrupt traumatic memories after retrieval because these memories are most certainly strong and in many cases have persisted for some time. In fact, many PTSD patients may take years to seek treatment, and chronic PTSD is

often the most difficult to treat. Another consideration is that memory retrieval happens outside of the clinical context, often in the form of re-experiencing of the traumatic event.

Replaying the traumatic event over and over again can sensitize patients with fear-related disorders and lead to worsening of the disorder. As in sensitization in humans with fear related disorders, animal studies have also shown that repeated retrieval can strengthen fear memory and make it impervious to disruption with treatments that normally disrupt memory reconsolidation (Inda *et al*, 2011). Thus, even if a drug is given each time a patient re-experiences a traumatic event, it may not be sensitive to disruption.

Future directions

Further areas of interest that are less well developed include studies of generalization versus discrimination, avoidance behavior and combined extinction-reconsolidation processes. The use of more sophisticated behavioral techniques in the laboratory to understand how fear generalizes to stimuli not originally associated with the traumatic event, which is a hallmark of PTSD and panic disorder, may provide powerful insight. An approach to studying generalization is to use differential fear conditioning whereby, in addition to a cue that signals shock, the animal is presented with a cue that is not followed by shock. Studies have shown that in rats (Duvarci and Varan, 2000) some animals show good discrimination, whereas others generalize fear to the safe cue, similarly to what is seen in patients with fear-related disorders. Another approach is to use conditioned inhibition training to identify animals that do not inhibit fear in the presence of a safety signal (Jovanovic *et al*, 2013). Both of these strategies can address a potential limitation of animal studies: the variability of responses is often not factored into the analyses, even though in people who experience a traumatic event there is great variability in responses, with some developing a pathological disorder and others being resilient (Yehuda and LeDoux, 2007). Also, early life stress and traumatic experience are factors that contribute to the development of PTSD. Yet there are relatively few preclinical studies determining the effects of early life trauma and stress on fear learning and fear extinction. More refined protocols are needed to model this important aspect of susceptibility to developing PTSD.

Another line of research that could potentially be relevant for the treatment of fear-related disorders is based on recent behavioural studies (Monfils *et al*, 2009; Schiller *et al*, 2010) demonstrating that, if extinction training occurs shortly after a single retrieval trial, fear memories are diminished and show no evidence of recovery. Although findings are not always consistent,

the ability to diminish fear memories in this manner opens another potential avenue by which traumatic memories can be targeted in patients with fear-related disorders.

Aim I

Histaminergic neurons are primarily located in hypothalamic tuberomamillary nucleus and send projections to the whole brain. Histamine controls several homeostatic functions and behavioral responses such as sleep, aversive memory formation and feeding behavior (Panula and Nuutinen, 2014). The involvement of central histamine in memory was initially observed by Almeida and Izquierdo (1986) as enhanced memory consolidation of the inhibitory avoidance (IA) paradigm after post-training i.c.v. infusions of histamine. Studies have shown the involvement histamine in different types of memory, related to fearful stimuli (Cangioli *et al.,* 2002; Benetti *et al.,* 2012), but also in memory impairment caused by maternal deprivation (Benetti *et al.,* 2014; 2012) or in memory consolidation of objects recognition (de Silveira *et al*., 2013).

Benetti's (Benetti *et al*, 2015) findings indicate that the histaminergic system comprises parallel, coordinated pathways that provide compensatory plasticity when one brain structure is compromised and that histamine is crucial in IA consolidation, depending on timing and on the brain area.

Mechanism that underlie memory retrieval are largely unknown but the growing interest on this process in psychiatry, in particular in behavioural therapies characterized by an alteration of the emotional spectrum, such as PTTD, prompts us to focus con aversive memory recall.

Here we investigate the role of the central histaminergic system in consolidation and in retrieval of IA memory via behavioural, pharmacological and biochemical techniques.

Materials and methods I

Animals

For behavioural procedure male Wistar rats (3 mounths old, 300–330 g) purchased from Centro de Reprodução e Experimentação de Animais de Laboratorio of the Universidade Federal do Rio Grande do Sul (our regular provider) were used. They were housed four to a cage with water and food ad libitum, under a 12:12h light-dark cycle (lights on at 0700-1900 h). The temperature of the animal room was maintained at 22–24 °C. All procedures were in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals, 1985) and were approved by Pontifical Catholic University of Rio Grande do Sul.

For biochemical experiments male Wistar rats (3 mounths old, 300–330 g) purchased from Envigo (Bresso, Italy). Animlas were housed in the animal facility of NEUROFARBA-Section of Pharmacology and Toxicology, Università di Firenze, housed in a temperature-controlled room (22 ± 1°C) with a 12:12-h light-dark cycle (light on 0700–1900 h), at a constant temperature and humidity with standard diet (4RF21; Mucedola s.r.l., Milan, Italy) and freely available water. All procedures were conducted in accordance with the Council Directive of the European Community (2010/63/EU) of the Italian Decreto Legislativo 26 (13/03/2014), and National Institutes of Health guidelines on animal care and approved by veterinarian supervision.

Surgery

At least 1 week after their arrival, rats were anaesthetized (75 mg/kg ketamine plus 10 mg/kg xylazine) and placed on a stereotaxic frame (Stellar; Stoeling). A stainless steel cannula (22 gauge) was implanted in the LV and fixed to the skull by using dental cement, according to the following coordinates: anterior, −0.9 mm; lateral, −1.5 mm; ventral, −2.6 mm (Paxinos and Watson, 1998), and used for a-FMHis administration. Rats were also implanted bilaterally with 22-gauge guide cannulae 1 mm above the CA1 area of the hippocampus, 1 mm above the BLA and 1 mm above vmPfCx. The coordinates were anterior, −4.2 mm; lateral, ±3.0 mm; ventral, −1.8 mm for the hippocampal CA1, anterior, −2.4 mm; lateral, ±5.1 mm; ventral, −7.5 mm for the BLA and anterior +3.2 mm; lateral ±0.8; ventral −2 mm for the vmPfCx (Paxinos and Watson, 1998). Animals were allowed 7 days to recover from surgery before behavioural and biochemical procedures. Animals were handled once daily for three consecutive days, and all behavioural procedures was conducted between 8:00 and 11:00 AM.

Correct Cannula Placements

Correct cannulae placement were verified by infusing a 4% (wt/vol) methylene blue solution over 30 s into the CA1 and vmPfcx region (1 µL per side) or the BLA (0.5 µL per side). The second day after the last behavioural procedure. Animals were killed 30 min later by an overdose of the anaesthetics; brains were withdrawn and stored in formalin. The spread of the dye was taken as an estimate of that of the drug infusions in the same animals. Placements were considered correct when the spread was 1 mm3 or less (de Carvalho Myskiw *et al.,* 2013; Fiorenza et al., 2012) from the intended infusion sites, which occurred in 98% of the animals. Only behavioral data from these animals were analyzed. As explained in Myskiw et al., (2008), despite the uncertainties given by the unknown rate of solubility of the drugs used relative to methylene blue, this procedure is an improvement over the mere determination of the cannula tip location.

Inhibitory Avoidance Task

The apparatus consisted in a 50 × 25 × 25 cm Plexiglas box with a 5-cm-high, 8-cm-wide, 25-cm-long Formica platform on the left end of a grid of 1-mm caliber bronze bars spaced 0.8 mm apart. The rats were gently placed on the platform facing the left rear corner. When they stepped down, placing their four paws on the grid, they received a 2-s 0.5-mA scrambled foot shock and then were immediately withdrawn from the training box. Retention test was carried out 48 hours after the training session. During test, the procedure was the same except that the foot shock was omitted. In the retention test, the step-down latency was 300 s. Latency to step down was measured with an automated stopwatch.

Chemicals

The drugs used were α-FMHis (5 µg/uL) was synthesized at Abbott Laboratories, histamine (10 mM), 2-2-pyridylethylamine (10 mM), dimaprit (10 mM), pyrilamine (50 mM) purchased from Sigma–Aldrich.

Infusion Procedure

At the time of drug microinfusions, the animals were gently restrained by hand, and the injection needle (30 gauge) was fitted tightly into the guides, extending 1 mm from the tip of the guide cannulae. The injection needle was connected to a 10-µL Hamilton microsyringe, and the infusions were performed at a rate of 0.5 µL/30 s. The infusion cannula was left in place for an additional 60 seconds to minimize backflow. It was then carefully withdrawn and placed on the other side, where the procedure was repeated. The entire bilateral infusion procedure took approximately 90 seconds. α-FMHis was infused into the LV 24 hours before the training and/or the test session.

Histamine was infused into the hippocampal CA1, BLA and vmPfcx 10 minutes before the test session, as well as pyrilamine, 2-2-pyridylethylamine and dimaprit into the hippocampal CA1. The volume of drugs infused was 0.5 µL per side in the BLA and 1 µL per side into the CA1 area, vmPfcx area and LV. Control groups received equal volumes of sterile saline (0.9%).

pCREB Experiments and Western Blotting Analysis

For the experiments aimed at determining pCREB levels, Male Wistar rats received infusions of saline or a-FMHis through a cannula into the lateral ventricle (LV) 24 hours before test, therefore divided into two experimental groups:

- Animals infused in LV with sterile saline, control group
- Animals infused in LV with a-FMHis

Animals from all groups immediately returned to their home cage after manipulations and were killed 10 minutes after training. After sacrifice, rat brains were dissected out on ice and amygdala, PfCx area and CA1 region of hippocampus immediately were isolated. The pooled structures (left and right) were individually homogenized in 200 µL of ice-cold lysis buffer containing protease and phosphatase inhibitors [50 mM Tris·HCl (pH 7.5), 50 mM NaCl, 10 mM EGTA, 5 mM EDTA, 2 mM sodium pyrophosphate, 4 mM p-nitrophenyl phosphate, 1 mM Na3VO4, 1.1 mM PMSF, 20 µg/µL leupeptin, 50 µg/µL aprotinin, 0.1% SDS) and centrifuged at 13.8 × g at 4 °C for 15 min. The supernatant was collected and total protein levels were quantified by using the Pierce BCA Protein Assay (Thermo Scientific). Homogenates were diluted in a mix of lysis buffer and loading buffer 2× (50 mM Tris pH = 6.8, 100 mM DTT, 10% Glycerol, 1% Bromophenol blue, and 2% SDS) and boiled for 10 min. Aliquots containing 50 µg of 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). Blots were blocked in Tris-buffered saline, pH 7.6 containing 0.1% of Tween 20 (TBS-T) and 5% nonfat dry milk (Bio-Rad Labora- tories) for 2 h at room temperature and then incubated overnight at 4 °C with monoclonal antibodies against phospho-CREB (Ser133) or CREB (both from Cell Signaling Technology) di- luted 1:1,000 in TBS-T containing 5% BSA or 5% nonfat dry milk, respectively. Immunodetection was performed with secondary antibodies (anti-rabbit IgG conjugated to horseradish peroxidase; Cell Signaling Technology) diluted 1:5,000 in TBS-T containing 1% of nonfat dry milk. Membranes were washed in TBS-T, and then reactive bands were detected by using enhanced chemiluminescence (Luminata Crescendo; Millipore). Quantitative densitometric analysis was performed by using the QuantityOne analysis software (Bio-Rad). For each sample, a ratio of pSer133-CREB/CREB densities

was calculated and then all of the individual rates were expressed as a percentage of the average of ratios obtained from control group.

c-Fos signle-staining

90 minutes after session, to fully assess *c-Fos* expression, rats were deeply anaesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with cold physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were post-fixed in the same solution overnight (4 ºC), and cryoprotected in 30% sucrose in PB. Forty µm thick sections were cut on a cryostat and collected in PB. Sections were preincubated in 0.75% H2O2 in PB for 30 min, in 0.2% BSA for 30 min and then incubated overnight in rabbit *c-Fos* primary antibody (1:5000; Sigma-Aldrich) at 4 ºC. The immunoreactive product was detected with the avidin-biotin peroxidase system (Vectastain kit; Vector Laboratories). After washing, sections were mounted on gelatin-coated slides, dehydrated, cover-slipped and observed using an Olympus BX40 microscope equipped with a Nikon DS-F1 camera. *c-Fos* immunopositive nuclei were counted bilaterally using the Image J software (NIH, USA) on 4-5 sections per region/per mouse and normalized to a 1 mm2 area according to Munari et al. (2013). Atlas coordinates relative to bregma (Franklin and Paxinos, 1998) for the sections analysed were from −3,8 mm to −4,4 mm for hippocampal CA1; from −2,12 mm to −2,75 for the BLA; from 2,7 mm to 1,95 for the vmPfcx. All regions analysed receive histaminergic fibres. Statistics were calculated on the average values from 4-5 sections of individual for each animal.

Data and Statistical analysis

Statistical analysis was performed using Prism Software (GraphPad Software). Data are expressed as means ± SEM. Inhibitory avoidance latencies as well were analysed with unpaired *t* test or one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. *c-Fos* expression and pCREB/CREB ratio were analysed with unpaired *t* test. *P* values less than 0.05 were considered statistically significant. The number of rats per group is indicated in the figure legends.

Results I

Central histamine modulates inhibitory-avoidance retrieval via activating hippocampal H1 but not H2 histaminergic receptors

Acute Depletion of Histamine Impairs the Recall of IA Memory Independently of Histamine Presence in Consolidation

We tested the performance of rats treated with α-FMHis infused 24 hours before or after the training session in the one-trial step-down IA. Controls received i.c.v. infusions of an equivalent volume of saline. The retention test was carried out 48 hours after training. As shown in figure xx all experimental groups had comparable step-down latency during training. One-way ANOVA analysis on retention latencies revealed a significant difference across groups ($F_{2,41}$= 62,89; P<0.0001). No differences in training performances were found in any examined group (Fig. I 1). Fourty-eight hrs after training, saline treated rats showed increased step-down latency indicative of acquired memory. However, a Bonferroni's post hoc test showed that step-down latency of all α-FMHis-treated groups (whether 24 hours before or after training session) were significantly shorter than that of the saline treated group (P<0.000; Fig. I 1). It is conceivable that rats receiving a-FMHis 24 hrs after training lacked integrity of the histaminergic system during retrieval and not during consolidation, whereas the opposite is true for rats receiving a-FMHis 24 hrs prior training, since a-FMHis effects on histamine levels persisted for about 48 hrs (Benetti et al., 2015). Memory consolidation relies on active and differentiated molecular events occurring at different times and different brain areas involved in modulation of mnemonic processes (Izquierdo, 2006; Izquierdo 1997; Bambah-Mukku, 2014): amnesia caused by α-FMHis 24 hours prior to training session indicates that histamine is one of the modulators of consolidation (Benetti et al., 2015). The present finding, confirm the earlier observation that central histaminergic system crucially impacts on IA by impairing consolidation (Benetti et al., 2015) and indicate that histamine influences retrieval of the aversive memory, independently of consolidation, by engaging active mechanisms in memory retrieval

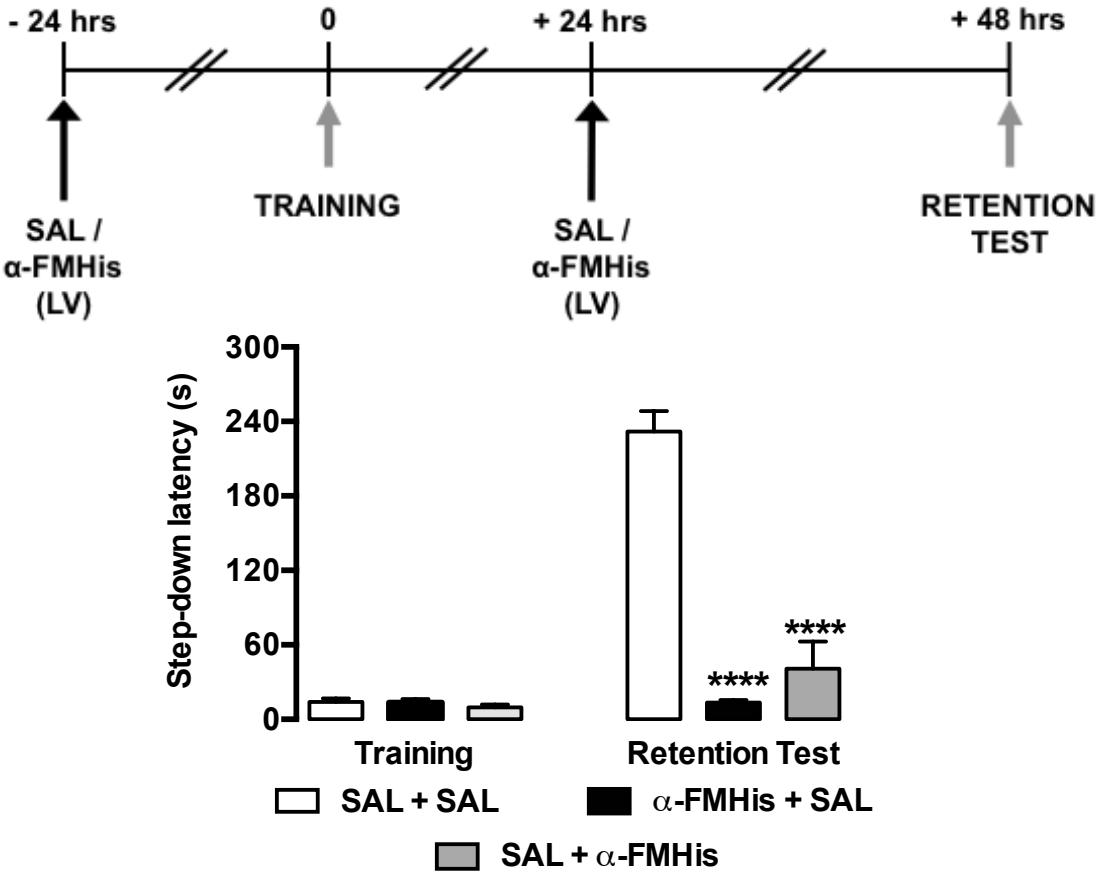

Figure I 1. Effect of the acute depletion of histamine caused by α-FMH i.v.c. infusion on consolidation and retrieval of IA task

The schematic drawing above the graph shows the sequence of experimental procedures and treatment administrations. Rats implanted with an infusion cannula in the LV received α-FMH or saline 24 hrs prior to or after the training session. The retention test was carried out 48 hrs after the training session. Step-down latencies of all groups that received α-FMH i.c.v. 24 before or after training session were significantly lower than controls (SAL + SAL). Data are expressed as means ± SEM of 10-12 animals for each experimental group; **** P<0.0001 vs. controls; one-way ANOVA followed by Bonferroni's MCT.

Effect of histamine in CA1 hippocampal region administration on cognitive impairments produced by α-FMHis administration 24 hours prior to retention test, but not prior to training

To further test the hypothesis of histamine modulating active mechanisms in IA retrieval, we investigated whether the administration of histamine in brain regions crucial for memory processing reverted the α-FMHis-induced amnesia. Histamine (10 mM) was infused 10 minutes before retention test bilaterally into the hippocampal CA1 region of rats given α-FMHis 24 hours prior to or after training. Controls received equal volumes of saline. Retention test was carried out 48 hours after training. No differences in latencies were found during training across groups. One-way ANOVA performed on retention latencies displayed a significant difference between groups ($F_{2\text{-}27}$=34.11; P<0.0001). Bonferroni's MCT showed that the step-down latencies of rats infused with α-FMHis 24 hours before training and with histamine 10 min before retention test were significantly shorter as compared to controls. However, animals treated with α-FMHis 24 hours after training and histamine before the retention test were not different from the step-down latency of controls infused with saline (Fig.I 2), thus suggesting that both groups of animals formed a memory trace of the training experience.

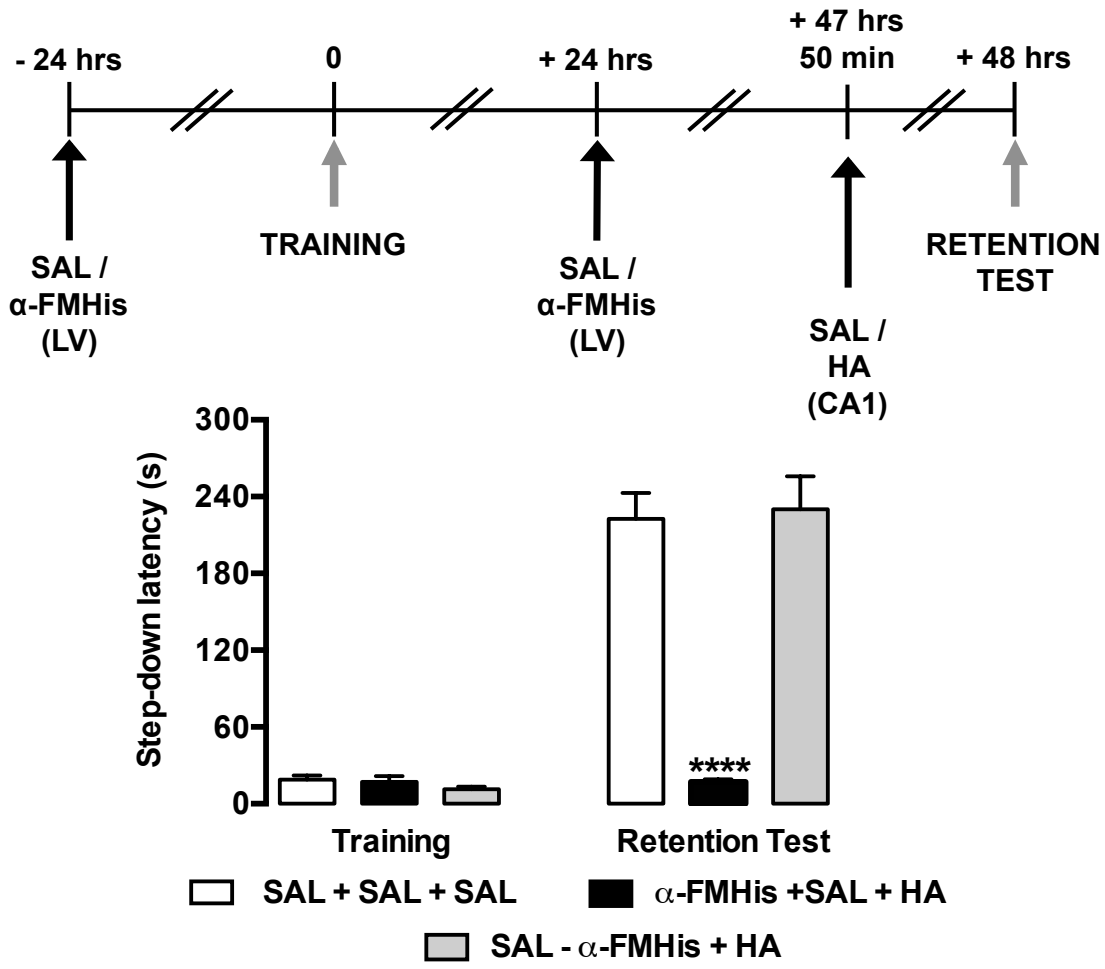

Figure II 2. Effect of intra-CA1 infusion of HA on amnesia caused by α-FMH i.c.v. infusion.

The schematic drawing above the graph shows the sequence of experimental procedures and treatment administrations. Rats were implanted with infusion cannulae in the LV and hippocampal CA1 bilaterally. α-FMH were injected i.c.v. 24 hrs prior or after the training session, and intra-CA1 histamine 10 min before the retention test. Rats receiving saline into both the LV and BLA served as controls. Data are expressed as means ± SEM of 10-12 animals for each experimental group; **** P<0.0001 vs. controls; one-way ANOVA followed by Bonferroni's MCT.

Histamine Infusion in BLA Prior to the Retention test does not revert Amnesia Caused by α-FMHis administration 24 hours prior to or after training

Previous work from our laboratory showed that exogenous administration of histamine after training either in hippocampal CA1 or BLA of rats acutely deprived of histamine during IA consolidation, hence amnesic, restored LTM with a different time frame, indicating that LTM could be formed if histamine transmission was restored up to 110 min after training in the BLA, and up to 6 hrs after training in the CA1 region of the hippocampus (Benetti et al., 2015), thus confirming the crucial role of BLA and CA1 in consolidating emotional memories (Lalumier, 2014). To test the involvement of the BLA in IA retrieval, we investigated whether administration of histamine (10 mM) counteracts the amnesic effect of α-FMHis. Histamine was given bilaterally into the BLA 10 minutes prior to retention testing of rats given α-FMHis 24 hrs prior to or after training. Controls received equal volumes of saline. Retention test was carried out 48 hours after training. No differences in latencies were found during training across groups. One-way ANOVA revealed a significant difference across groups ($F_{2,24}$=59.97; P<0.0001). Further Bonferroni's MCT showed that all rats treated with α-FMHis, independently whether before or after training, and with intra-BLA histamine displayed latencies significantly shorter than controls (Fig. I 3; p<0.001). Thus, histamine given into the BLA, in this time frame, did not antagonize the amnesic effect of α-FMHis administered prior to the retention test.

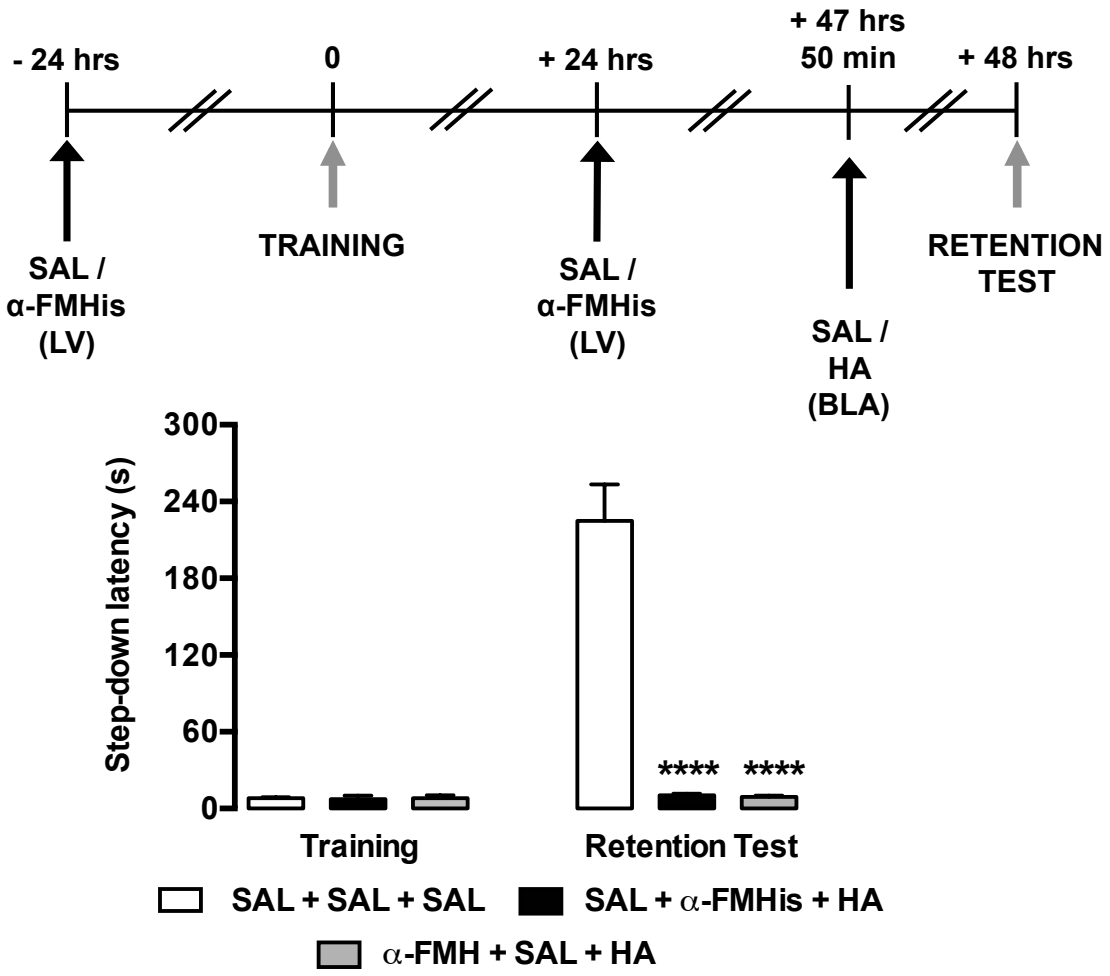

Figure I 3. Effect of intra-BLA infusion of HA on amnesia caused by α-FMH i.v.c. infusion.

The schematic drawing above the graph shows the sequence of experimental procedures and treatment administrations. Rats were implanted with infusion cannulae in the LV and BLA bilaterally. α-FMH was injected i.c.v. 24 hrs prior to or after the training session, and intra-BLA histamine (10 nM) 10 min before the retention test. Rats receiving saline into both the LV and BLA served as controls. The retention test was carried out 48 hours after the training session. Data are expressed as means ± SEM of 10-12 animals for each experimental group; **** $P<0.0001$ vs. controls. one-way ANOVA followed by Bonferroni's MCT.

Histamine Infusion in the Ventromedial Prefrontal Cortex (vmpfCX) Prior to the Retention Test does not revert Amnesia Caused by α-FMHis administration 24 hours prior to or after training

Convergent evidences from human and rodent studies support a role for the vmpfCX in the regulation of fear memory expression, especially enhancing the ability to form associations between aversive threats and their predictors (Arruda-Carvalho and Clem, 2015). To test the involvement of histaminergic transmission in the vmpfCX in IA retrieval, we investigated whether administration of histamine (10 mM) counteracted the amnesic effect of α-FMHis. Histamine was infused bilaterally into the vmpfCX 10 minutes prior to testing retention of rats given α-FMHis 24 hrs prior to or after training. Controls received equal volumes of saline. Retention test was carried out 48 hours after training. One-way ANOVA performed on the retention test revealed a significant difference across groups ($F_{2,40}$=41.05; P<0.0001). No differences in latencies were found during training across groups. Further analysis with Bonferroni's MCT showed that all rats treated with α-FMHis, independently of the timing (before or after training) and with histamine displayed latencies significantly shorter than controls (Fig. I 4). Thus, histamine given into the vmpfCX did not antagonize the amnesic effect of α-FMHis administered prior or after the retention test.

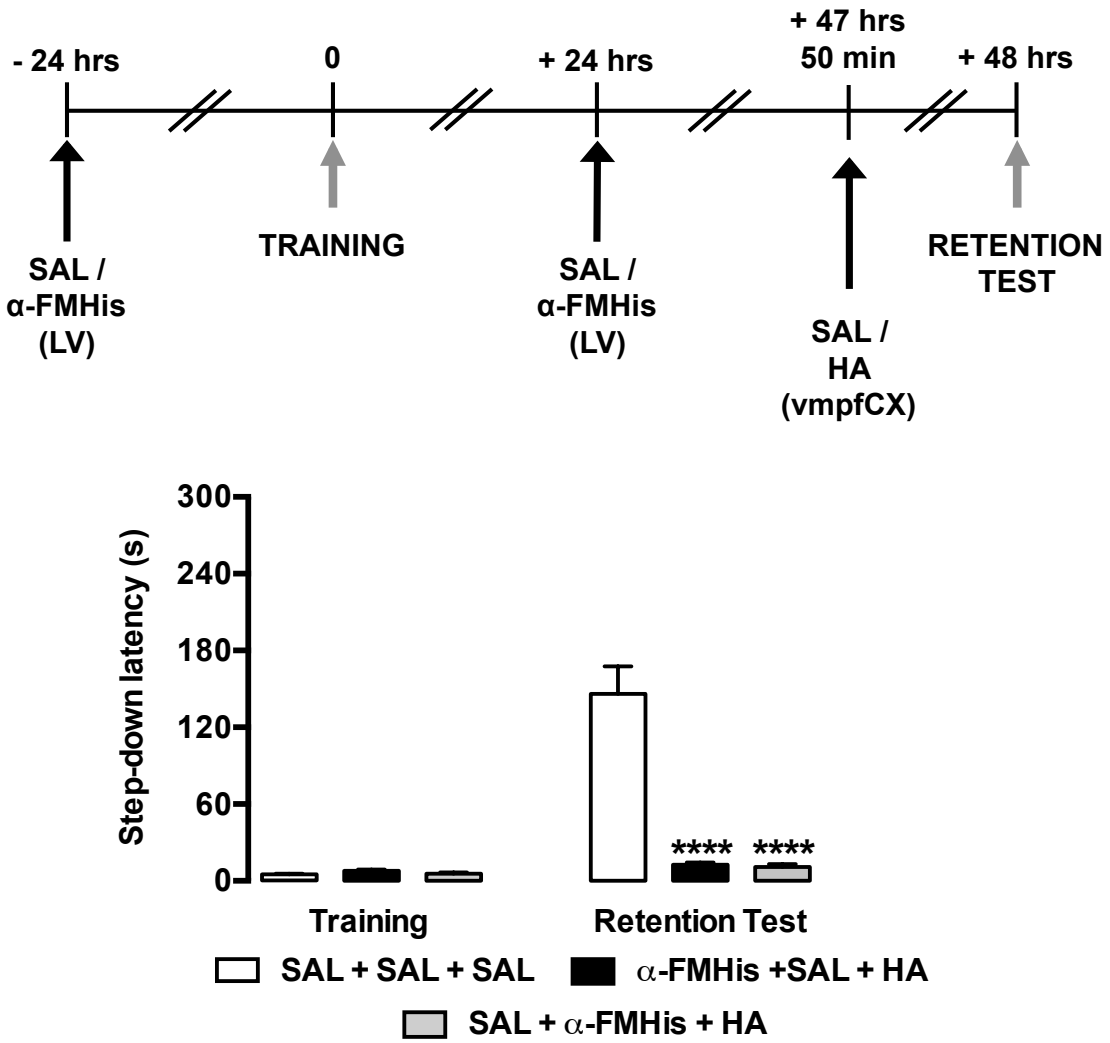

Figure I 4. Effect of intra-vmpfCX infusion of HA on amnesia caused by α-FMH i.c.v. infusion.

The schematic drawing above the graph shows the sequence of experimental procedures and treatment administrations. Rats were implanted with infusion cannulae in the LV and vmpfCX bilaterally. Rats received α-FMH i.c.v. 24 hrs prior to or after training session and intra-vmpfCX histamine (10 mM) 10 min before the retention test. Rats receiving saline into both the LV and vmpfCX served as controls. The retention test was carried out 48 hrs after the training session. Data are expressed as means ± SEM of 10-12 animals for each experimental group; **** $P<0.0001$ vs. controls. one-way ANOVA followed by Bonferroni's MCT.

Administration of histamine into the CA1 failed to prevent impairment of IA retrieval induced by anisomycin infusion immediately after training

The consolidation of a LTM trace requires transient changes in the activity of intracellular signalling cascades that regulate new gene transcription and de novo protein synthesis in the brain (Davis and Squire, 1984; Helmstetter et al., 2008; Johansen et al., 2011; Jarome and Helmstetter, 2014). Consistent with this, protein synthesis inhibitors impair LTM for a variety of behavioural tasks when infused into the brain around the time of training (Jarome and Helmstetter, 2014). Because histamine bilaterally infused into the CA1 prior to test session counteracted α-FMHis amnesia only when this was administered 24 hrs prior to but not after training, but not before training, we hypothesize that rats formed a memory trace of the training experience when histamine was present 24 hours after training, but were not able to retrieve it if histamine neurotransmission was abrogated during the retention test. Because protein synthesis associated with consolidation have been implicated in memory consolidation (Szapiro et al., 2002; Murchison et al., 2004), we investigated whether bilateral intra-CA1 infusion of histamine could induce the recall of the memory trace in rats that received intra-CA1 infusions of the protein translation inhibitor anisomycin (80 μg/μl) 10 minutes after training. Histamine (10 mM) was infused 10 min before the retention test. Controls received equal volumes of saline. Retention test was carried out 48 hours after training. One-way ANOVA revealed no differences in latencies were found during training across groups. One-way ANOVA performed on the retention test revealed a significant difference in step-down latencies across groups ($F_{3,44}$=160.7; P<0.0001). Further analysis with Bonferroni's MCT showed that all groups treated with anisomycin immediately after training session, independently of histamine, showed shorter step-down latencies compared to controls treated with saline (Fig. I 5). These results corroborate the hypothesis that retrieval relies on protein synthesis carried out during the consolidation process and demonstrate that intra-CA1 histamine administration prior to the retention is capable of reverting α-FMHis-induced amnesia when this is administered after completion of consolidation (24 hrs after training), but cannot revert the amnesia caused by protein synthesis inhibition during consolidation.

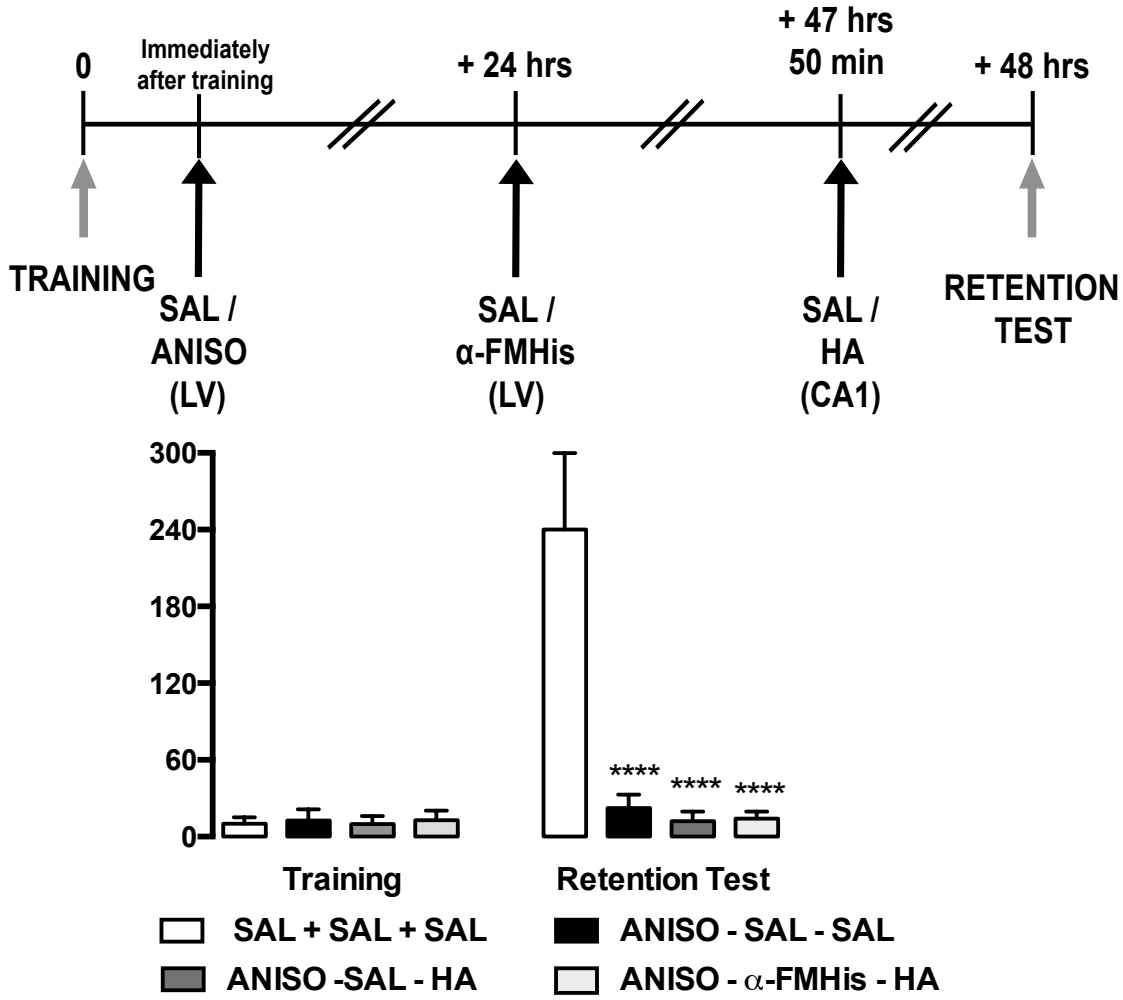

Figure 5. Histamine administration did not revert the amnesic effect produced by administration of anisomycin immediately after training

The schematic drawing above the graph shows the sequence of experimental procedures and treatment administrations. Rats were implanted with infusion cannulae in the LV and hippocampal CA1 and received α-FMH i.c.v. 24 hrs after the training session, intra-CA1 anisomycin immediately after the training session and intra-CA1 histamine 10 min minutes before the retention test. Rats receiving saline into both the LV and CA1 served as controls. Data are expressed as means ± SEM of 10-12 animals for each experimental group; **** $P<0.0001$ vs. controls; one-way ANOVA followed by Bonferroni's MCT.

Activation of Histaminergic H1 but not H2 Receptor Subtype in the CA1 Counteracts the Amnesia Caused by α-FMHis Administration 24 Hours Prior to the Retention Test

Histamine modulates several phases of different types of memory, mostly activating H2 receptor subtype (Gianlorenco et al., 2015; Furini et al., 2014; da Silveira et al., 2013; Giovannini et al., 2003). We then investigated which receptor subtype mimic the effect of histamine in hippocampal CA1. The H1R-agonist 2-2-pyridylethylamine (PEA; 10 mM) or the H2R-agonist dimaprit (DIM; 10 mM), was infused bilaterally into the hippocampal CA1 region in rats given α-FMHis 24 hours after training. Controls received equal volumes of saline. Retention test was carried out 48 hours after training. No differences in latencies were found during training across groups. One-way ANOVA performed on the retention test revealed a significant difference in step-down latencies across groups ($F_{2,49}$=19.99; P<0.0001). No differences in latencies were found during training across groups. Further analysis with Bonferroni's MCT showed that only PEA-treated rats recovered the memory trace, as demonstrated by the comparable step-down latencies of this group to controls that did not receive a-FMHis (Fig. I 6). However, the latencies of DIM-treated animals resulted significantly shorter compared to the control group (P<0.001), therefore DIM did not revert α-FMHis-induced amnesia.

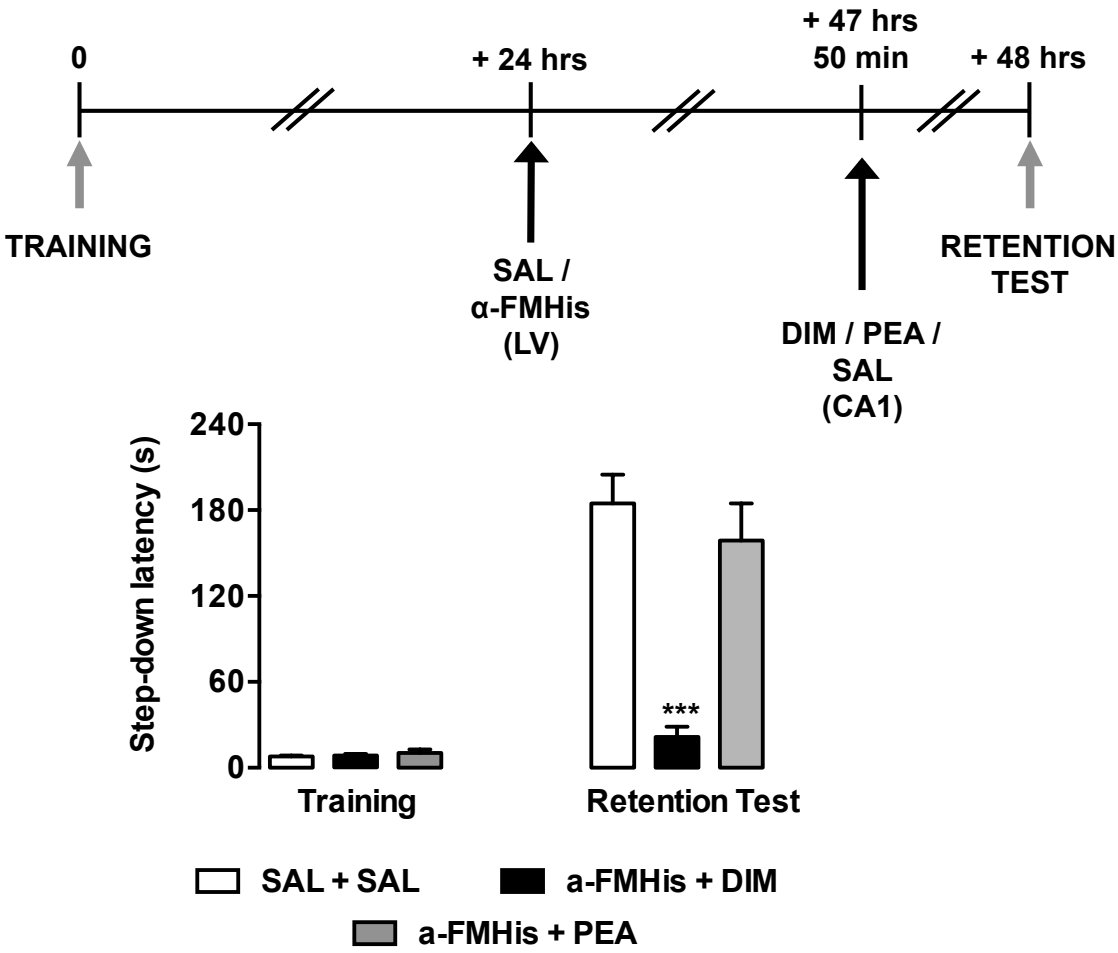

Figure 6. Effect of different histaminergic agonists infusion in the CA1 on amnesia caused by α-FMH i.c.v. infusion. The schematic drawing above the graph show experimental procedures and treatment administration. Rats were implanted with infusion cannulae in the LV and hippocampal CA1 and received α-FMH i.c.v. 24 hrs prior to or 24 hrs after training session. The H1R agonist 2-2-pyridylethylamine or the H2R agonist dimaprit were injected intra-CA1 10 minutes after training session. Rats receiving saline into both the LV and CA1 served as controls. The retention test was carried out 48 hours after training session. Data are expressed as means ± SEM of 10-12 animals for each experimental group; **** P<0.0001 vs. controls; one-way ANOVA followed by Bonferroni's MCT.

Effect of pyrilamine intra-CA1 infusion on IA-retrieval

We demonstrated that intra-CA1 infusion of the H1 receptor agonist PEA reverted α-FMHis-induced amnesia, therefore we tested whether the infusion of a selective H1 antagonist could inhibit IA-retrieval. Rats underwent IA training and 10 minutes before the retention test they were infused with the H1 receptor antagonist pyrilamine (50nM) bilaterally in the hippocampal CA1 region. Controls received equal volumes of saline. The retention test was carried out 48 hours after training. No differences in latencies were found during training across groups. One-way ANOVA performed on the retention test revealed a significant difference in step-down latencies across groups. Rats given intra-CA1 pyrilamine 10 minutes before the retention test showed shorter step-down latencies compared to controls (controls = 123 ± 46.0 s vs. pyrilamine = 13.6 ± 7.85; $P<0.05$; unpaired *t* test; Fig. I 7). These results further confirm the involvement of the H1 receptor subtype in controlling IA-retrieval.

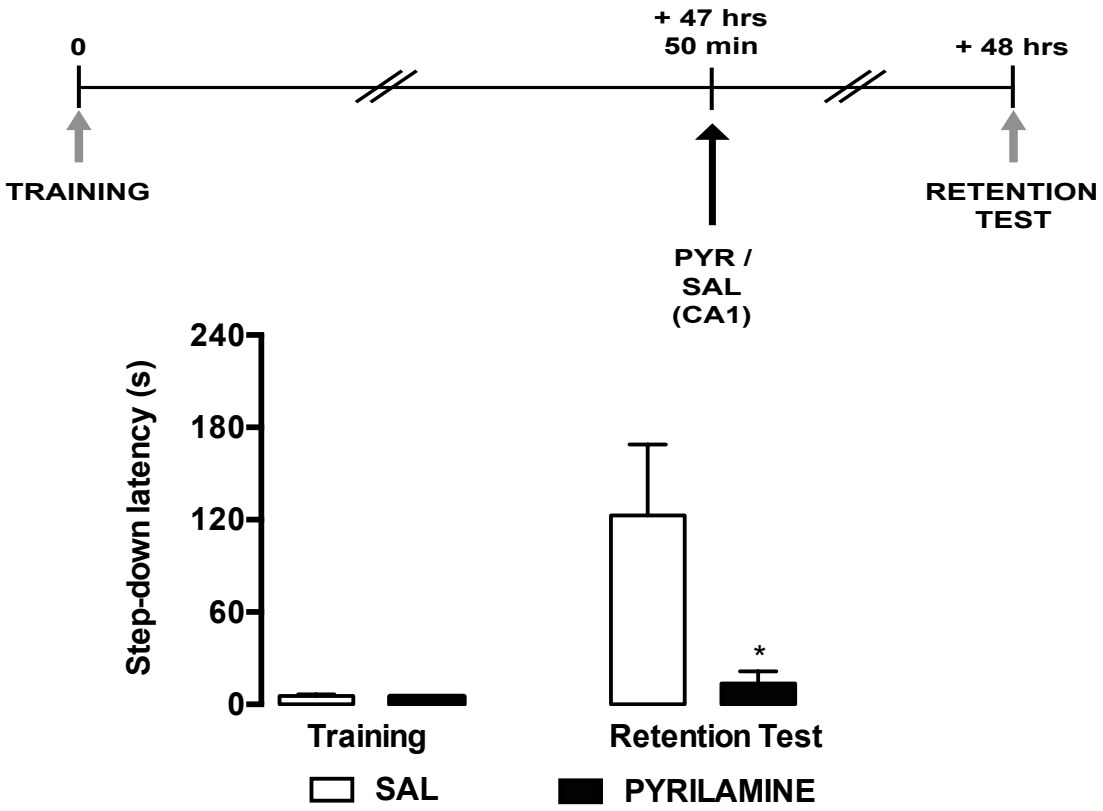

Figure I 7. Effect of intra-CA1 infusion of the H1R antagonist pyrilamine on IA-recall.

The schematic drawing above the graph shows the sequence of experimental procedures and treatment administration. Rats were implanted with infusion cannulae bilaterally in CA1 and received pyrilamine (50 mM) 10 min before the recall test. controls received equal volumes of saline. Data are expressed as means ± SEM of 10-12 animals for each experimental group; * $P<0.05$; unpaired *t* test.

Effect of histamine acute depletion on *c-Fos* expression and CREB phosphorylation after retrieval in the CA1 region of the hippocampus

To understand which biochemical mechanisms are inherent in IA-retrieval and whether the acute lack of histamine could modify them, we used c-Fos expression, a marker of functional activity, and we evaluated cyclic adenosine monophosphate (cAMP) responsive-element-binding protein (CREB) phosphorylation inasmuch an increased CREB phosphorylation at Ser-133 in the hippocampus is specifically associated with IA memory formation (Bernabeu et al., 1997; Viola et al., 2000). Benetti and colleagues (2015) reported that histamine-induced CREB phosphorylation in the BLA and hippocampus correlated to its pro-mnemonic effect in IA-consolidation. Increased pCREB expression was absent in α-FMHis treated rats. Rats received α-FMHis or saline i.c.v. 24 hrs before the training session, while retention test was carried out 48 hours late. As shown in the photomicrographs (Fig. I 8A), rats treated with α-FMHis 24 hours prior to test displayed a lower activation of the dorsal hippocampus, as demonstrated by the lower number of c-Fos immuno-positive nuclei compared to controls (control = 88.87 ± 8.7 per square millimeter vs. α-FMHis-treated = 33.17 ± 3.8 per square millimeter; $P<0.01$, unpaired *t* test). For Western Blot analysis rats have been euthanized 10 minutes after retention test. As shown by densitometric analysis of immunoblots (Fig. I 8B), rats given α-FMHis 24 prior to test presented a decreased pCREB density compared to saline treated animals (control = 99.33 ± 1.9 pCREB density vs. α-FMHis-treated = 60.00 ± 7.3; $P<0.01$; unpaired *t* test).

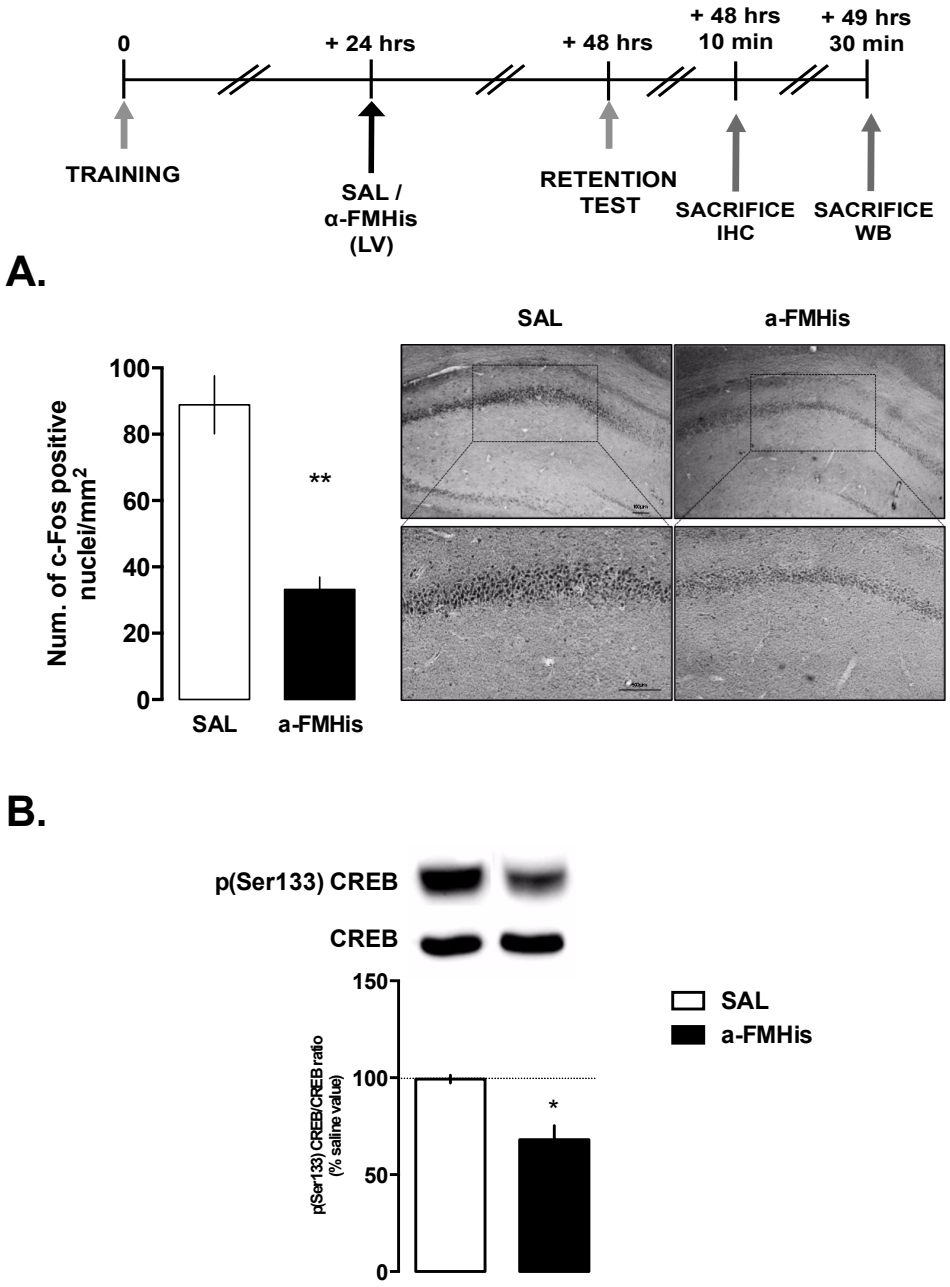

Figure I 8. Effect of the acute central histamine depletion during IA-retrieval on *c-Fos* activation and CREB phosphorylation in the dorsal hippocampus.

The schematic drawings above the graphs show experimental procedures, treatment administration and time of sacrifice. Rats were implanted with a cannula in the LV and received α-FMH 24 hours after the training session; controls received equal volumes of saline. In the panel A, representative microphotographs show c-Fos immune-positive nuclei in the CA1 of rats treated with α-FMH compared to controls (Scale bar: 100 μ). Data are expressed as means ± SEM of 3-4 rats for each experimental group; controls = 88.87 ± 8.7 per square millimeter vs. α-FMHis-treated = 33.17 ± 3.8 per square millimeter; ** P<0.01, unpaired *t* test). In panel B, representative immunoblots and densitometric analysis of % pCREB/CREB ratio in the dorsal hippocampus of α-FMH-treated rats compared to controls. The pCREB/CREB ratio is expressed as percentage with respect to controls of 4 rats for each experimental group.

Effect of histamine acute depletion on *c-Fos* expression and CREB phosphorylation after retrieval in the BLA

BLA is essential in IA-consolidation, with vicarial role when the activity of the dorsal hippocampus is impaired (Benetti et al., 2015). We therefore analysed the activation and CREB phosphorylation in the BLA. Rats received α-FMHis or saline i.c.v. 24 before the training session, while retention test was carried out 48 hours later. As shown in figure I 9A no difference in BLA activation was found across groups, as demonstrated by the comparable number of *c-Fos* immuno-positive nuclei of α-FMHis-treated rats with controls. No statistical differences were found as well in pCREB/CREB in the BLA homogenates across groups (Fig. I 9B).

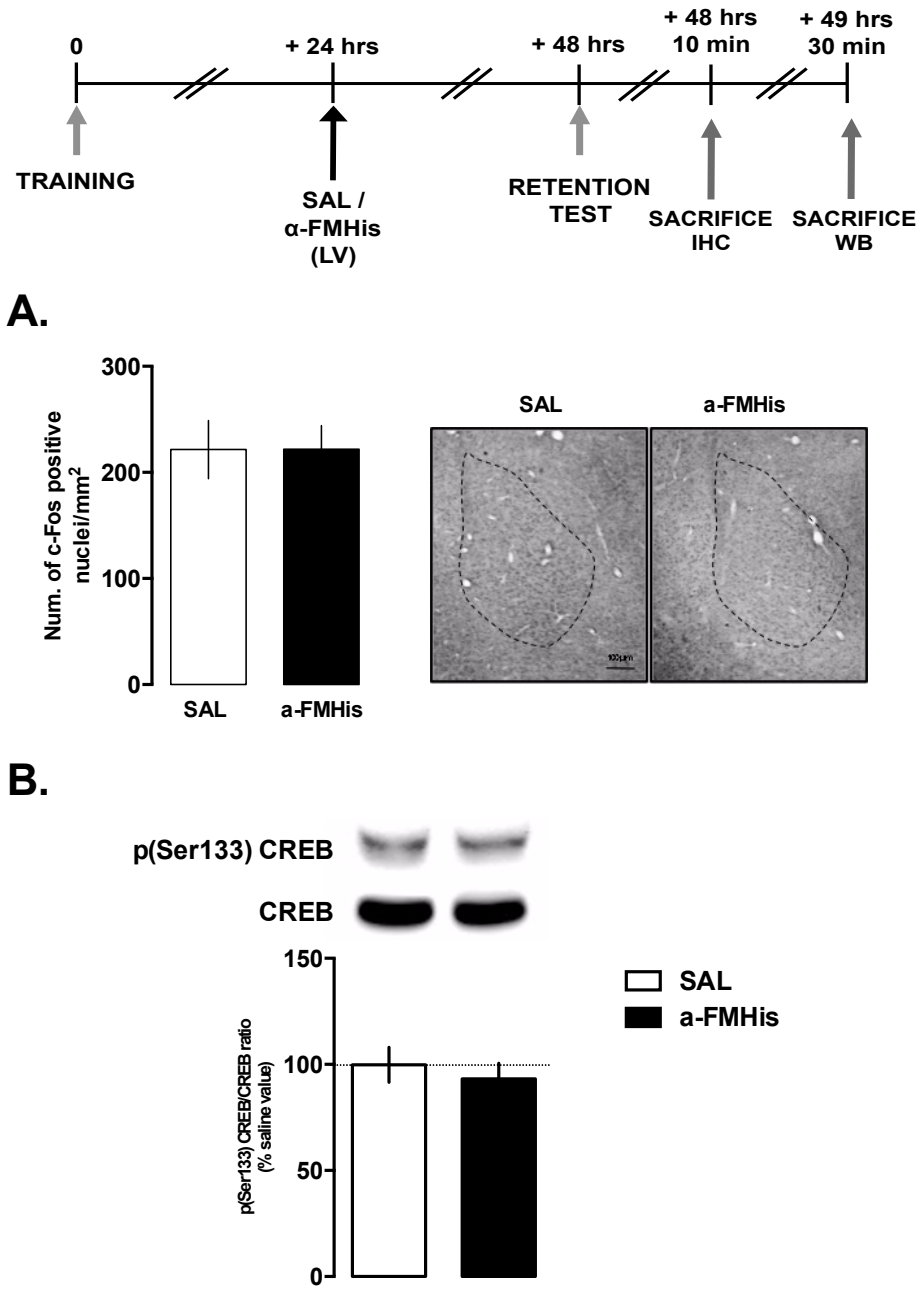

Figure I 9. Effect of the acute depletion of central histamine during IA-retrieval on c-Fos activation and CREB phosphorylation in the BLA.

The schematic drawings above the graphs show experimental procedures, treatment administration and time of sacrifice. Rats were implanted with a cannula in the LV and received α-FMH 24 hours after IA-training session; controls received equal volumes of saline. In the panel A, representative microphotographs show c-Fos immune-positive nuclei in the amygdala of rats treated with α-FMH compared to controls (Scale bar: 100 μ). Data are expressed as means ± SEM of 3-4 rats for each experimental group; controls = 221.5 ± 27.32 per square millimeter vs. α-FMHis-treated = 219.8 ± 22.39 per square millimeter). In panel B, representative immunoblots and densitometric evaluation of pCREB/CREB ratio in the amygdala of α-FMH-treated rats compared to controls; pCREB/CREB ratio are expressed as percentage in respect to controls of 4 rats for each experimental group.

Effect of histamine acute depletion on *c-Fos* expression and CREB phosphorylation after retrieval in the ventromedial prefrontal cortex

Rats received α-FMHis or saline i.c.v. 24 before the training session, while retention test was carried out 48 hours late. As shown in figure I 10A no difference in vmpfCX activation was found across groups, as demonstrated by the comparable number of c-Fos immuno-positive nuclei of α-FMHis-treated rats compared to controls. No statistical differences were found in pCREB/CREB in the vmpfCX homogenates across groups (Fig. I 10B).

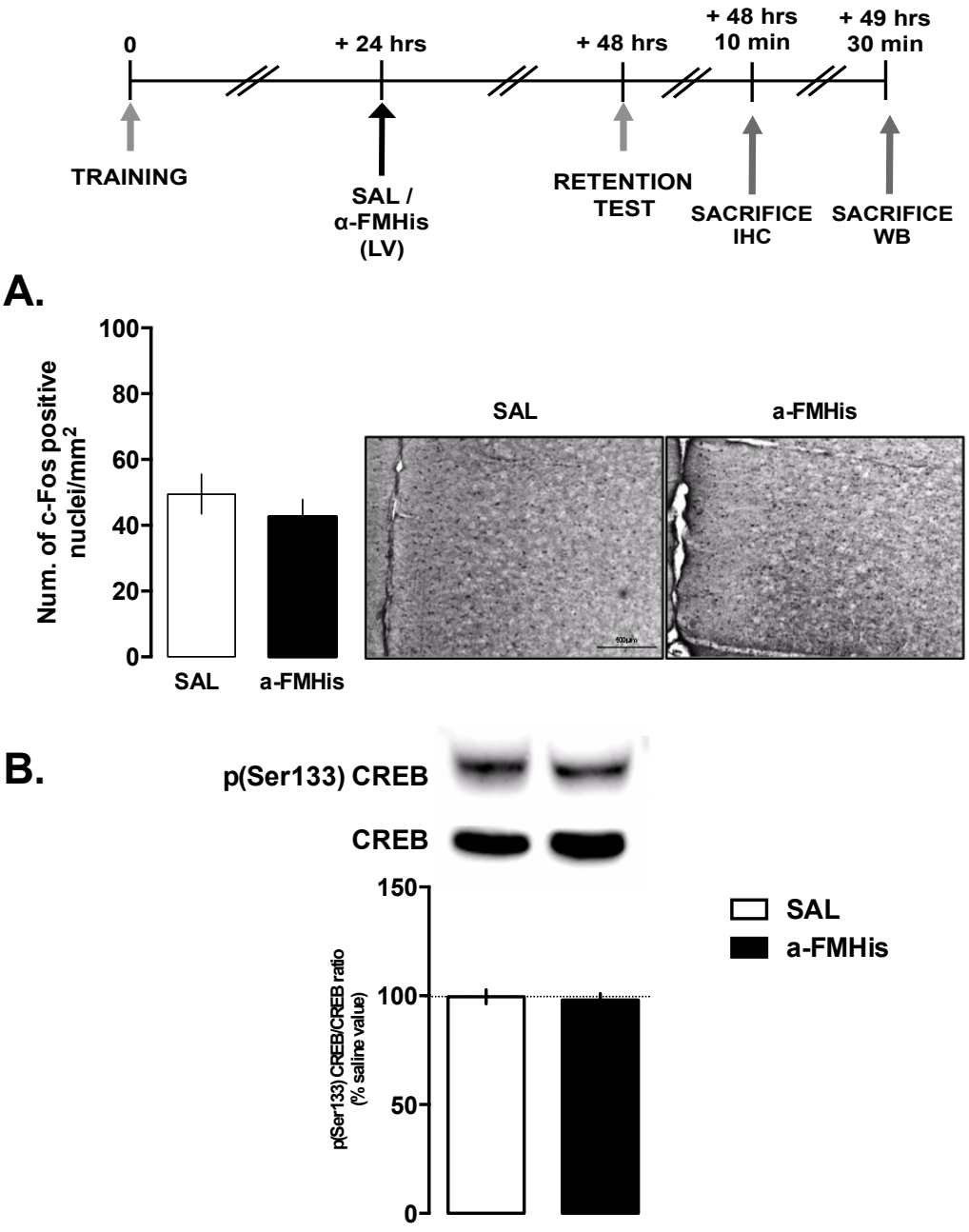
0
+ 24 hrs
+ 48 hrs
+ 48 hrs
10 min
+ 49 hrs
30 min
TRAINING
SAL /
α-FMHis
(LV)
RETENTION
TEST
SACRIFICE
IHC
SACRIFICE
WB
A.
Num. of c-Fos positive
nuclei/mm2
100
80
60
40
20
0
SAL
a-FMHis
SAL
a-FMHis
B.
p(Ser133) CREB
CREB
150
100
50
0
p(Ser133) CREB/CREB ratio
(% saline value)
SAL
a-FMHis

Figure I 10. Effect of the acute lack of central histamine during IA-retrieval on *c-Fos* activation and CREB phosphorylation in the prefrontal cortex.

The schemes above the graphs show experimental procedures, treatment administration and time of sacrifice. Rats were implanted with a cannula in the LV and received α-FMH 24 hours after IA-training session; controls received equal volumes of sterile saline. In the panel A, representative microphotographs show c-Fos immune-positive nuclei in the prefrontal cortex of rats treated with α-FMH compared to controls (Scale bar: 100 μ). Data are expressed as means ± SEM of 3-4 rats for each experimental group; controls = 99.80 ± 8.26 per square millimeter vs. α-FMHis-treated = 93.20 ± 7.30 per square millimeter; ns; unpaired *t* test). In panel B, representative immunoblots and densitometric evaluation of pCREB/CREB ratio in the vmpfCX of α-FMH-treated rats compared to controls; pCREB/CREB ratio was expressed as percentage with respect to controls of 4 rats for each experimental group.

Conlcusion I

Central histaminergic system is critically involved in modulation of basic homeostasis and higher functions such as arousal, circadian and feeding rhythms, and cognition (Köhler *et al*, 2011; Panula and Nuutinen, 2013). The first insight of the involvement of central histamine in modulating memory dates back to 1986 when de Almeida and Izquierdo described how the immediate post-training i.c.v. administration of histamine in rats facilitates retention test performance of step-down inhibitory avoidance measured 24 hours later. Recent studies described histamine as a fundamental physiological modulator in different memory paradigms such as novel object recognition (da Silveira *et al*, 2013; Giovannini *et al*, 1999), contextual fear conditioning (Baldi *et al*, 2005; Cangioli *et al*, 2002; Giovannini *et al*, 2003; Passani *et al*, 2001), social recognition (Esbenshade *et al*, 2012; Prast *et al*, 1996) and memory impairment caused by early maternal deprivation (Benetti et al., 2015). Pathologically, histaminergic system degeneration seems to have a role in different neuropsychiatric disorders in human, among them also Tourette syndrome (Rapanelli and Pittenger, 2015). Memory encoding requires changes in neuronal activity of different areas across the brain (Köhler *et al*, 2011) and the histaminergic system stands as one the major of these modulatory systems. Recently, Benetti and colleagues (2015) highlighted that the recruitment of alternative brain circuits allows compensation of memory impairment following damage to brain regions specialized in integrating and /or storing specific memories. First they reported that the integrity of the central histaminergic system is crucial in consolidation of long-term but not short-term memory of step-down inhibitory avoidance (IA) paradigm. The same authors linked phosphorylation of cyclic adenosine monophosphate (cAMP) responsive-element-binding protein a crucial mediator in long-term memory formation, anatomically and temporally with histamine-induced memory processes, showing the active involvement of histamine function in CA1 and BLA in different phases of memory consolidation, and indicating that the histaminergic system comprises parallel, coordinated pathways that provide compensatory plasticity when one brain structure is compromised (Benetti *et al*, 2015). It is now well known and described that memory consolidation consists of complex neuronal rearrangements and active processes (Squire, 1982) but less is known about the mechanisms underlying retrieval of memory: how are specific brain areas able to reassemble learned information stored and codified in brain networks back into the language we use to communicate? Retrieval, the use of learned information, was until recently mostly "*terra incognita*" in the neurobiology of memory, due to shortage of research

methods with the spatiotemporal resolution required to identify and dissect fast reactivation or reconstruction of complex memories in the mammalian brain (Ben-Yakov *et al*, 2015).

Memories are encoded as enduring physical changes in the brain, or engrams (Semon, 1921; 1923). There is agreement between scientists about the fact that the formation of engrams involves the strengthening of synaptic connections between discrete populations of neurons but the exact nature and location of engrams has been challenging. Lashley was the fist to attempt to locate engrams with empiric methods (Franz and Lashley, 1917; Lashley, 1950): his research went unsuccessfully and his conclusion that engram is receding. Today, we understand that this fleeing understanding of engram's nature was due, in part, to the widely distributed and dynamic nature of memory representation in the brain, making engrams challenging to identify.

Starting from the definition of memory as the ability of individuals to acquire, store and recall information based on experience, the term engram was introduced by Semon more than a century ago (Semon, 1921; 1923) and defines the physical substrate of memory in the brain, trace characterized by four features (Schacter *et al*, 2001): persistence, ecphory, content and dormancy. First, the engram is a long-lasting change in the central nervous system that results from a specific experience. The engram has the potential for ecphory, an automatic recall process roused by a specific cue interactions with information stored: so the engram could be expressed behaviourally through interactions with retrieval cues, which can be sensory, ongoing behaviours or directed and voluntary goals. Third, its content reflects what transpired during the encoding and predicts what can be recovered during subsequent retrievals, and last, an engram can exist in a dormant state between the two active processes of encoding and retrieval. Therefore the engram is not memory itself but provides the necessary physical conditions for memory to emerge (Josselyn *et al*, 2015; Schacter *et al*, 2001). The most accepted point of view is that the formation of an engram needs strengthening of synaptic populations (Bliss and Collingridge, 1993; Kandel and Schwartz, 1982; LeDoux, 2014; Scoville and Milner, 1957). Engrams are not confined to a single brain region but rather may be composed by different networks (Josselyn *et al*, 2015). It is also important to note that the engram is not static: after encoding, consolidation process, although this is a fixation or stabilization of a memory, may alter the physical and chemical organization of engrams, which could lead to a qualitative and quantitative modification of the engrams; as well, memory recall can transiently make labile a previously consolidated engram and start another consolidation cycle (Dudai, 2002; Winocur *et al*, 2010). Even if engrams change target over time, this characteristic does not impair the possibility to find and "capture" them over at different moments (Josselyn *et*

al, 2015). Finally, different memory types may be due to engrams in distinct collections of brain area (Josselyn *et al*, 2015), depending on timing, stimulus and behavioural cue.

Ramon y Cajal was the first to speculate that there are specific experience-induced changes at level of the neuron and, even if he was convinced that neurons do not change after development, he hypothesized that neurons were modifiable by experience, in a process that he called "cerebral gymnastics" (RAMON Y CAJAL, 1952). Afterward Hebb proposed that the process of learning strengthened the synaptic connections between neurons, facilitating the formation of neuronal assemblies (Milner, 1999). These neuronal assemblies are thought to comprehend collections of neurons that fire together at the time of learning and, sometimes with modifications, during recall and they were proposed as the neuronal substrate of the engram (Josselyn *et al*, 2015).

Empirical studies demonstrated that environmental enrichment (Volkmar and Greenough, 1972), learning (Moser *et al*, 1994) and long-term potentiation (Muller *et al*, 2000) alter brain structure at the level of spine morphology. Other persistent learning-induced modification have been described such as changes in DNA structure (Day and Sweatt, 2011), post-translational modification of kinases

(Sacktor and Fenton, 2012), activation of transcription machinery (like CREB phosphorylation; (Josselyn and Nguyen, 2005)), induction of immediate-early genes (IEGs), such as Fos (Guzowski, 2002), phosphorylation and trafficking of receptors, alteration of synaptic strength and changes in neural excitability (McKernan and Shinnick-Gallagher, 1997). Indeed in the brain the life of the engram is extremely dynamic: all the changes already described are not predictable of a secure recall of a consolidated memory and even a well-consolidated engram is changeable: studies showed that a "dormant" engram in the brain is well protected from potential erasing but when the trace is recalled or retrieved it can be made labile and modifiable (Baldi and Bucherelli, 2015). As in all memories, fear memory recall of a consolidated engram is a dynamic (Baldi *et al*, 2015), active and stand-alone process, characterized by its own modification in brain structure, areas activation and biochemical processes.

In the present study the recall of IA memory in rats acutely deprived of central histamine by i.c.v. infusions of α-FMH, an irreversible histidine decarboxylase inhibitor, that is able to fully suppress spontaneous and evoked histamine release from histaminergic neurons. The present finding confirms Benetti et al., (2015) earlier observation that central histamine depletion crucially impacts on IA by impairing long-term consolidation and indicates that histamine influences retrieval of the aversive memory, independently of consolidation, by engaging active mechanisms in memory retrieval. As central histamine was depleted in retrieval but not during consolidation,

rats were not able to show a manifestation in retention test of the consolidated memory, although that happened in the presence of histamine. Memory retrieval requires the activation of several brain areas, critically involved even in consolidation (Izquierdo and McGaugh, 2000) and our results demonstrate that the presence histamine in hippocampal CA1 area is necessary to recall the previous consolidated information, confirming the active nature of this process. The engram was stored, in a specific temporal frame, in the hippocampus and to prove that what we evaluated infusing histamine in CA1 in retention test was the memory trace, we administered a protein synthesis blocker, anisomycin, that unable consolidation therefore the building of the engram, before histamine's infusion in CA1: consolidation protein synthesis was impaired and histamine did not revert α-FMH-induced amnesia, because no mnemonic trace was codify and consolidated. Secondly, we described how the presence of histamine in BLA and vmpfCX in IA memory retrieval was not necessary within the time window explored in the present experiments. As Benetti and co-workers (2015) described, the involvement of histamine in IA memory consolidation strictly depends on timing and topographical histaminergic activation, leading us to speculate that, following the temporary early activation of the BLA in consolidation, histamine support IA retrieval by acting in the hippocampus. Reports studying the specific contribution of histaminergic receptor subtype are focused on consolidation and extinction and highlighted the critical role H2-Rs (Benetti and Izquierdo, 2013; da Silveira *et al*, 2013; Furini *et al*, 2014; Giovannini *et al*, 1999). Surprisingly, the present results demonstrate that IA retrieval relays on the activation of hippocampal H1-Rs but not H2-Rs subtype. Clinically, classical antihistamines, normally used in allergies, and especially the first generation, beside the sedation, also induce mild cognitive impairments (Tannenbaum *et al*, 2012). When we infused pyrilamine, a first-generation antihistamine, clinically used in treating allergies, in hippocampal CA1 10 minutes before IA retention rest, we saw an impairment in the recall of the memory trace acquired and consolidated, confirming the critical role of H1-Rs in retrieval of aversive memory. The connection between CREB and long-term memory was further established in several organisms, using methods that enabled disruption of CREB function. Induction of a dominant-negative mutation or deletion of key CREB isoforms blocked long-term memory in *Drosophila* and in mice (Bourtchuladze *et al*, 1994). The mechanisms that underlie the ability of CREB to facilitate memory are not completely understood – they could involve processes such as the induction of long-term potentiation or depression of synaptic strength, the growth and formation of new synaptic connections (Marie *et al*, 2005), or protein synthesis-dependent processes involved in the retrieval and reconsolidation of memory (Kida *et al*, 2002). Therefore we here studied biochemical

modification induced by the acute lack of histamine in IA retrieval: as a matter of fact, in IA retrieval CREB phosphorylation was reduced in hippocampal CA1 in animals acutely deprived of brain histamine compared to controls that goes along with a stronger CA1 activation, as demonstrates via c-Fos analysis. No difference in the levels of CREB phosphorylation between controls and a-FMHis-treated animals was observed In the BLA nor in the vmpfCX,. These results indicate that the histaminergic transmission in the CA1 is crucial for IA memory retrieval in this temporal phase and occurs despite the blockade of histaminergic neurotransmission in the BLA and the vmpfCX.

Taken together our present results suggest the crucial involvement of central histamine not only in IA consolidation (Benetti *et al*, 2015) but also in IA retrieval. The histaminergic control of IA retrieval seems to relay in the hippocampus via activating H1Rs and inducing CREB phosphorylation in the dorsal hippocampus but not in the BLA nor in the vmpfCX, in this time window. Importantly, these findings describe the active nature of memory retrieval, pointing out that mechanisms that underlay consolidation and retrieval are not identical. An overgrowing interest on retrieval in exposure therapies of memory impairments, such as post-traumatic stress disorders (PTSD), prompts us to propose the central histaminergic system as a potential target in the treatment of emotional disorders.

CHAPTER II

BRAIN HISTAMINE AND FEEDING BEHAVIOUR

Mechanisms controlling hungry and satiety

Definitions of Satiation and Satiety

Satiation refers to the postprandial feeling of fullness that may be one of pleasure or distress, accompanied by other symptoms such as fullness, nausea, or bloating. Operationally, satiation can be defined as the maximum tolerated volume of a liquid nutrient meal, and by intraprandial and postprandial symptoms experienced with the meal challenge. Satiety reflects the appetite to ingest meals, and it may be defined operationally by the kilocalories ingested at a subsequent ad libitum buffet meal after a standard period of fasting or a specified time from a prior standard meal, typically ingested 4 hours previously (eg, a 300-kcal meal). It is relevant to distinguish these 2 terms because satiation reflects the early postprandial experience, whereas satiety reflects the appetite to ingest food at a subsequent meal after a period of fasting. Because obesity results from the imbalance between energy consumed and energy expended, postprandial satiation and satiety are critically relevant to the development of obesity (Camilleri and Acosta, 2015).

Gut-Brain Communication

The vagal nuclei and the vagus nerve innervate most of the gastrointestinal tract involved in energy intake, satiation, and digestion (Korner and Leibel, 2003). Vagal afferents are stimulated directly by changes in viscus tension when food, or later chyme, passes through the gut gastrointestinal tract, and indirectly by chemical stimuli activating taste receptors, releasing peptides from mucosal enteroendocrine cells. Some of these peptides act on vagal or other pathways to induce appetite (an orexigenic effect; e.g. ghrelin), whereas others (e.g. gastric leptin, CCK, GLP-1, or peptide tyrosine-tyrosine) induce satiety (Camilleri *et al*, 2015). Circulating nutrients, reflecting levels of nutrients in the periphery, influence brainstem nuclei after being sensed in the area postrema in the floor of the fourth ventricle, where there is a thin blood-brain barrier (Fry and Ferguson, 2007). In response, the brain stem controls the enteric nervous system (Berthoud, 2008), modulating upper-gut function or signalling to the hypothalamic circuits to reduce calorie intake. On the other hand, partial vagotomy, total subdiaphragmatic vagotomy, or intermittent vagal nerve electrical stimulation to inhibit vagal function in humans (Camilleri, 2008) decrease food intake and induce early satiety and weight loss, possibly by reducing gastric emptying and inducing satiation.

Mechanisms Regulating Appetite: Hypothalamic and Brainstem Mechanisms

Several interacting control mechanisms that involve peripherally released mediators are involved in the regulation of appetite (Korner *et al*, 2003). Hypothalamic circuits involve several peptide receptors that control appetite and food intake. These mechanisms include cannabinoids NPY, pro-opiomelanocortin, melanin-concentrating hormone, α-melanocyte stimulating hormone, agouti-related peptide, cocaine- and amphetamine-regulated transcript, CCK, and GLP-1 (Camilleri *et al*, 2015). Neural pathways link the hypothalamic nuclei to higher centres, which produce food reward or a feeling of well-being, and to the brainstem nuclei. Through the brainstem nuclei, such as the NTS and the dorsal motor nucleus of the vagus, the hypothalamus can slow gastric emptying by stimulating vagal fibres that activate intramural gastric nitrergic neurons to decrease gastric motility (Berthoud, 2008) retard gastric emptying, and, as a consequence, decrease calorie consumption (Ste Marie *et al*, 2000). The precise pathways and centres involved in rewards appear to involve NPY and dopaminergic receptors (Huang *et al*, 2001) and eating for reward value is a primitive behaviour observed in species such as *Drosophila* (Wang *et al*, 2014). Preliminary data in human beings using magnetic resonance imaging (MRI) with pulsed arterial spin labelling in response to a nutrient drink (Ensure; Abbott Laboratories, Chicago, IL) show that the sensation of satiation is associated with decreased cerebral blood flow in the hypothalamus compared with a control brain region (posterior frontal cortex). By using repeated measurements at 15-minute intervals, hypothalamic pulsed arterial spin labelling MRI signal decreased significantly after ingesting the maximum tolerated volume of the liquid meal, and this decreased signal persisted 30 minutes later (Abu-Dayyeh *et al*, 2014). The effect of nutrients in Ensure may reflect the greater reduction in cerebral blood flow in the hypothalamus to the monosaccharide glucose compared with fructose ingestion (Page *et al*, 2013) as well as increased functional connectivity between the hypothalamus and the thalamus and striatum with glucose, and increased connectivity between the hypothalamus and thalamus, but not the striatum, with fructose. In another study, the blood oxygenation level–dependent signal in the cortical control areas increased during glucose infusion and decreased during fructose infusion (Purnell *et al*, 2011). The significance of these findings is unclear; however, fructose ingestion was associated with a smaller increase in systemic glucose, insulin, and GLP-1 levels than glucose ingestion (Page *et al*, 2013). The time course of changes in hypothalamic and brainstem fluctuations in the blood oxygenation level–dependent signal on functional MRI in response to sucrose (a combination of glucose and fructose) was consistent with a rapid, vagally mediated mechanism caused by nutrient absorption, rather than sweet taste-receptor activation (Kilpatrick *et al*, 2015). Gastrointestinal hormones including leptin,

cholecystokinin, PYY, oxyntomodulin, and GLP-1 may affect nuclei in the hypothalamus and brainstem, where the blood-brain barrier allows direct interaction between these hormones with the nuclei, just as described earlier for nutrients. The circulating peptides inhibit the agouti-related peptide/NPY pathway in the arcuate nucleus of the hypothalamus, thereby reducing appetite and stimulate the pro-opiomelanocortin/α-melanocyte stimulation hormone pathway, and increasing satiety and indirectly reducing appetite (Hillebrand *et al*, 2002).

Gut Peptidergic and Hormonal Control of the Response to Feeding and Satiation

Ingested nutrients and their digestion products initiate local actions in the upper gut, producing signals that initiate digestion and absorption. Other signals lead to the feeling of satiation, either directly or indirectly, through effects on gastric function, and lead to meal termination. Even during ingestion of the meal, gastric emptying of liquids results in the rapid delivery of nutrients into the intestine. Gastric and duodenal vagal afferents are stimulated by the mechanical, chemical, and osmotic effects of ingested nutrients, stimulating the release of a variety of peptides and hormones (Kirkup *et al*, 2012), which mediate or modulate digestion, sensation after feeding, and appetite (Strader and Woods, 2005). Ghrelin is an orexigen that is important in short-term food intake; in contrast, leptin is a minor orexigen, and obestatin is a peptide encoded by the ghrelin gene that opposes ghrelin's effects on food intake, delays gastric emptying, and inhibits jejunal motility (Zhang *et al*, 2005). CCK is a major mediator of satiation, providing negative feedback to the stomach (delaying emptying, at least in part, by fundic relaxation and antral inhibition). GLP-1 is an incretin that modulates glucose control and provides similar negative feedback to the stomach. Peptide YY is involved in appetite control, the ileal brake, and negative feedback to the stomach (Camilleri *et al*, 2015).

Adiposity- and Glycaemia-Related Hormones

Insulin from the pancreatic β-cells and leptin from white adipocytes, as well as the stomach and other tissues, each are secreted in direct proportion to body fat. Both hormones are transported through the blood-brain barrier (Banks, 2006; Woods *et al*, 2003) and access neurons in the hypothalamus and other regions of the brain to influence energy homeostasis. In contrast to satiation signals, which primarily influence calories eaten during individual meals, adiposity signals are related more directly to how much fat the body carries and maintains. Insulin systemically elicits hypoglycemia, which increases food intake. In obesity, there is insulin and leptin resistance; thus, more of each hormone is required to achieve the same physiological effect when compared with lean individuals (Camilleri *et al*, 2015).

Dysregulation of Gastric Function, Satiety, and Satiation Mechanisms in Obesity

Comparisons of gastric emptying in normal weight and obese persons have shown inconsistent results with rapid, normal, or slow gastric emptying (Park and Camilleri, 2005).

Autopsy studies in obese subjects have shown that some intra-abdominal organs such as the liver, small intestine, and pancreas are heavier than in normal-weight individuals (Naeye and Roode, 1970). Other studies showed wide variation of stomach size with no significant relationship to body weight (Cox, 1952). Obese subjects have been shown to choose more food and to consume more food per minute than non-obese subjects (Nasser *et al*, 2004). Obese people with binge eating disorder also show greater motor impulsivity (Nasser *et al*, 2004), choosing small, immediately available rewards over larger, delayed rewards, and/or the inclination to respond rapidly without forethought and/or attention to consequences (Swann *et al*, 2002). Gastric capacity was larger in obese persons with binge eating disorder when tested with an intragastric latex balloon filled with water (Gelibter *et al*, 2004). Other studies using the barostat or imaging single photon emission computerized tomography (SPECT) techniques reported no differences in gastric volume or compliance between obese and lean subjects (Vazquez Roque *et al*, 2006).

Recent studies in a prospective cohort of 328 participants across the spectrum of BMI from normal weight to class II or III obesity have shown that obesity is associated with higher fasting gastric volume, accelerated gastric emptying of solids and liquids, lower postprandial PYY, and higher postprandial GLP-1 levels (the latter being consistent with the accelerated gastric emptying of nutrients; (Acosta *et al*, 2015)). Obesity also was associated with delayed satiation, manifested as a larger volume of liquid nutrient ingested at a steady state to induce fullness (Acosta *et al*, 2015) and a larger maximum tolerated volume (Delgado-Aros *et al*, 2004); in addition, the total caloric intake at an *ad libitum* meal was greater in people with an abnormal (high) waist circumference (Acosta *et al*, 2015).

Fasting gastric volume influences intraprandial satiation, and the rate of gastric emptying also influences postprandial fullness (Delgado-Aros et al., 2004). Principal components analysis identified latent dimensions accounting for approximately 81% of overweight-obesity variation, including satiety/satiation (21%), gastric motility (14%), psychological (13%), and gastric sensorimotor (11%). These observations suggest that quantitative traits of satiation, satiety, and gastric functions are associated with a higher BMI (Acosta *et al*, 2015).

Gut-Brain axis: oleoylethanolamide from the intestine to the brain, a multifunctional fat sensor

Knowing the complexity of the interaction between stimuli that control feeding, it becomes clear that the ability to monitor the nutrient composition of food allows animals to generate neural and endocrine feedback signals that adapt behaviour and metabolism to environmental fluctuations in food availability: to sense dietary fat is critical, from an adaptive point of view, because of the high energy of this nutrient and the essential role of lipids in building cell membranes and precursors for hormones and other bioactive molecules (Piomelli, 2013). In the mouth, the first station, dietary fats are able to strongly affects feeding (Ackroff *et al*, 2010): in rodents, this step is sufficient to activate, as described above, taste-neurons in NTS, that project to subcortical areas of the central nervous system that control reward-oriented behaviour (Liang et al., 2006), and release of endocannabinoids in the gut (DiPatrizio *et al*, 2011), which is strongly correlated with the natural attraction to high-fat foods in mammals (Ackroff *et al*, 2010; DiPatrizio *et al*, 2011).

The infusion of lipid emulsions into the duodenum markedly suppresses food intake in both rodents and humans (Ackroff *et al*, 2010). In rats, this satiating action is abrogated by removal of the afferent nerves that connect the gastrointestinal tract to the brain(Sclafani *et al*, 2003), and is accompanied by activation of vagal afferents in the gut (Randich *et al*, 2000) and neurons in the NST (Zittel *et al*, 1994). More than 50 years ago, they were firstly isolated from plants and animal tissue (BACHUR *et al*, 1965) and it was described their anti-inflammatory action (Lambert *et al*, 2002).

With the growing interest for anandamide as an endocannabinoid , the appeal of other members of the FAE family increased leading to discover the function served by these molecules as regulators of food intake (Rodríguez de Fonseca *et al*, 2001) and, eventually, to identifying PPAR-α as a key cellular effector of this regulatory action. It also became apparent that the FAEs participate in a variety of physiological and pathological processes in addition to feeding – including pain (Calignano *et al*, 1998; Sasso *et al*, 2013), innate immunity (Lo Verme *et al*, 2005; Solorzano *et al*, 2009), and reward regulation and may thus represent a previously unrecognized class of multifunctional lipid mediators.

From the periphery to the central nervous system: the travel of OEA's anorexant signal

A high-fat diet promotes the formation of OEA in the different species, including rodents and humans (Petersen *et al*, 2006; Rodríguez de Fonseca *et al*, 2001). In rats three simultaneous events are responsible for this effect: a stronger production of OEA's precursor, the enhancement of OEA synthesis and the inhibition of its catabolism. Experiments in which individual nutrients were infused separately into the duodenum of catheterized rats showed that fat (Intralipid®) is a potent stimulus for jejunal OEA production, whereas sugar (glucose) and protein (Peptone®) have no such effect (Petersen *et al*, 2006; Schwartz, 2011). Only oleic acid but not palmitic one is able to increase OEA synthesis and this indicated that the presence of non-esterified ('free') oleic acid in the lumen of the small intestine stimulates mucosal cells to generate OEA (Schwartz, 2011). Studies with genetically modified mice revealed that deletion of CD36 abrogates food-induced OEA production, suggesting that this protein acts as a biosensor for food-derived oleic acid (Schwartz, 2011). CD36 plays an obligatory role in this process, but its precise functions are not entirely clear. In addition to promoting oleic acid uptake, as discussed above, CD36 might also regulate the activities of NAPE-PLD and FAAH, possibly by engaging Src-family protein tyrosine kinases, which are known effectors of CD36-dependent signalling in platelets (Huang *et al*, 2001). In rats and mice, intraperitoneal or oral administration of OEA induce a strong and long-lasting inhibition of feeding (Astarita *et al*, 2006; Fu *et al*, 2007; Gaetani *et al*, 2003; Rodríguez de Fonseca *et al*, 2001), importantly without causing any changes in fear or anxiety, corticosterone plasmatic levels or inducing taste-aversion, indicating that its hypophagic actions cannot be attributed to stress or malaise (Rodríguez de Fonseca *et al*, 2001). Rodents are nocturnal animals and they feed in series of meals with different duration and time-windows between bouts. OEA effect on feeding behaviour seems to depend on different aspects but, mostly, on the nutritional state of the animal: in rat fed *ad libitum*, the substance decreases meal frequency without altering meal size; by contrast, the compound simultaneously reduces these two parameters in food-deprived animals (Fu *et al*, 2007). But how does OEA exert its anorexant effect? Several studies support the idea that this effect is mediated by the activation of PPAR-α, a nuclear receptor: as a matter of fact OEA is able to bind the purified ligand-binding domain of PPAR-α in low concentrations and stimulates transcriptional activity of this protein (Fu *et al*, 2007), OEA analogues are not able or are less active compared to OEA, indicating a specific effect (Fu *et al*, 2007). Mice, genetically lacking PPAR-α, respond normally to other anorexic agents, but not to OEA (Fu *et al*, 2007). OEA activates the capsaicin receptor TRPV1 (transient receptor potential cation channel vanilloid-1),

but only when this receptor has been previously phosphorylated by protein kinase C (Ahern, 2003; Wang *et al*, 2005). In agreement with the established role of TRPV1 in the processing of painful stimuli, IP or intradermal injections of OEA evoke short-lasting nociceptive responses in wild type mice, but not in mice lacking TPRV1 (Lo Verme *et al*, 2005). OEA also acts as a medium-potency agonist for GPR119 (EC50 ~3 μM; (Overton *et al*, 2006)Overton et al., 2006), a G protein-coupled receptor that recognizes a broad panel of lipid molecules in addition to OEA. 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). The fact that dietary fat elevates OEA levels only in the upper gut suggests that this lipid mediator might also produce its anorexic effects through a mechanism mediated by the vagus nerve: as a prove, systemic, but not intraventricural administration reduces food intake (Rodríguez de Fonseca *et al*, 2001); indeed, local increases in small-intestine OEA production mimic the hypophagic effects of exogenous OEA (Fu *et al*, 2007). Further demonstrations are that a vagotomy prevents such effects, but not those of centrally acting anorexiants (Rodríguez de Fonseca *et al*, 2001), and the elevated doses of capsaicin, that causes transmission in peripheral vagal and non-vagal sensory fibres, also inhibited the anorexiant effect of OEA (Rodríguez de Fonseca *et al*, 2001). Most importantly, IP injections of OEA stimulate transcription of c-Fos (a marker of neuronal activation) in the NTS (Lo Verme *et al*, 2005; Rodríguez de Fonseca *et al*, 2001). Sclafani and colleagues described how surgically resecting the celiac-superior mesenteric ganglion (CSMG) complex, the sympathetic station that innervates with noradrenergic projections the intestine and other viscera, it is possible to interrupt the ability of fat in the duodenum to reduce food intake (Sclafani *et al*, 2003), this finding goes along with the fact that this same surgical procedure shortens the interval between meals, not only counteracting OEA effect on timing, but also preventing OEA production in the gut (Rodríguez de Fonseca *et al*, 2001). The finding that pharmacological antagonists of β2-adrenergic receptors also interrupt small-intestinal OEA mobilization provides further support for a permissive role of post-ganglionic sympathetic fibres in gut OEA signalling. IP OEA injections stimulate c-Fos expression in peptide-secreting neurons of the para-ventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (Gaetani *et al*, 2010; Rodríguez de Fonseca *et al*, 2001). Studies in rats have shown that systemic OEA administration causes an increase in the expression of the neuropeptide oxytocin, but not vasopressin, in magnocellular neurons of the PVN and SON (Gaetani *et al*, 2010). Moreover, pharmacological blockade of central oxytocin receptors abrogates the hypophagic effects of OEA, implying that release of oxytocin in the hypothalamus and/or other regions of the brain may be a key effector of

OEA-induced satiety (Gaetani *et al*, 2010). The identification of a functional link between OEA signalling in the gut and oxytocin transmission in the CNS raises the intriguing, but as-yet unexplored possibility, that OEA might also trigger other actions of oxytocin, such as facilitation of social behaviours (Insel and Young, 2001). Noradrenergic projections that connect the NST to the basolateral complex of the amygdala (BLA) are part of a neural circuit that is crucially implicated in the consolidation of recent emotional memories (McGaugh and Izquierdo, 2000). Considering that the ability to retain contextual information associated with nutrient sources would provide an adaptive advantage to animals foraging in the wild, it is plausible that OEA might reinforce such ability by strengthening memory consolidation (Campolongo *et al*, 2009). It appears, therefore, that OEA signalling in the gut initiates an integrated response in which satiety induced by a fat-rich meal coincides temporally with enhanced encoding of information about the spatial and emotional context in which the meal was consumed (Piomelli, 2013).

The central histaminergic control on eating behaviour

The first insight of the link between central histaminergic system and eating disorder became clear when is was observed that atypical antipsychotic drugs (AAPDs) increased appetite and weight gain: these effects are mediated by a selective and potent activation of hypothalamic AMP kinase, that regulates food intake and that reverses the action of the hypophagic agent leptin. These effects are prevented in animals with deletion of the H1 receptor (Kim *et al*, 2007); moreover, the potencies of AAPDs in blocking H1 receptors are correlated with their orexigenic properties (Kim *et al*, 2007).

Clineschmidt and Lotti provided the first direct description of the inverse correlation between brain histamine and appetite (Clineschmidt and Lotti, 1973): the administration of this amine that does not cross the blood brain barrier, into the lateral ventricle of cats induced a long-term inhibition of food intake. Similarly, not only the continuous infusion of histamine into the suprochiasmatic nucleus of the hypothalamus, but also its acute administration in the lateral ventricle (Lecklin *et al*, 1998) suppressed food intake in rats. As a further demonstration, comparable effects were described after systemic administration of the histamine precursor, L-histidine (Vaziri *et al*, 1997; Yoshimatsu *et al*, 2002) or of the inhibitor of histamine catabolism metopramine (Lecklin *et al*, 1998). Later it was found that the activation of H1 receptors in the PVN and VMH of the hypothalamus caused satiety (Masaki and Yoshimatsu, 2006). Coherently, the blockade of H3 auto- and eteroreceptor, mediating the release of histamine and other

neurotransmitters, affected has beneficial effects on different behavioural and metabolic parameters linked with food intake and eating behaviour (Provensi *et al*, 2015).

Central histaminergic system not only has a modulatory effect on food intake, but it also regulates feeding circadian rhythms. Infusing α-fluoromethylhistidine (α-FMHis, a suicidal blocker of histidine decarboxylase) in rat's third ventricle disrupts light-dark cycle that modulates feeding, drinking and ambulatory activity in animals fed *ad libitum* (Passani and Blandina, 2011). Food availability it's a powerful driving circadian stimulus and when food restriction occurs in a predictable time of the day, animals show increased food searching behaviours anticipating the meal, coherently with the state of raised arousal, and to the increased motivation to feed (Mistlberger, 1994). The involvement of central histamine in arousal induced by feeding was demonstrated by the cluster-specific activation of neurons belonging to the E3 sub-area of the TMN immediately before the exact time of meal in rats that underwent a scheduled feeding (Meynard *et al*, 2005; Umehara *et al*, 2011). Furthermore, histamine release increased in the posterior hypothalamic area when rats, subjected to food restriction, tried to open a mesh container filled with enticing food (Valdés *et al*, 2010). All these experimental settings highlight that the histaminergic activation parallels an arousal induced by the expectation of food, therefore strictly linked to the appetitive phase of feeding behaviour (Provensi *et al*, 2015). There exist evidences that indicate the involvement of central histamine in the consummatory phase of feeding, as described by the transient, but significant increase of histamine release in the hypothalamus when 24 hours-fasted rats were re-fed (Itoh *et al*, 1998).

Central histamine, as already mentioned, interacts with peripheral substances that control feeding behaviour. As a matter of fact, gastrointestinal tract and adipose tissue release more than 20 regulatory peptide hormones that influence different physiological processes and, in addiction to the local paracrine actions and peripheral endocrine effects, these modulators provide information on the nutritional status to the hypothalamus and brainstem, that integrate and elaborate the peripheral input with brain signals (e.g. reward and mood) and contribute to regulate hunger or satiety (Provensi *et al*, 2015). Provensi and colleagues (2014) recently described the crucial role of the histaminergic system in the hypophagic effect of OEA. As mentioned above, OEA exerts it action via activating sensory fibres in the vagus nerve, reaching the NTS, that projects noradrenergic fibres to the PVN and excites the hypothalamic oxytocin system (Romano *et al*, 2013). Provensi and co-workers then demonstrated a new participant in OEA's control of feeding behaviour, as in genetically and acutely histamine deprived mice (HDC-KO and i.c.v. injected with α-FMHis, respectively) OEA hypophagic effect was significantly diminished,

in terms of amount of food consumed. The authors speculated that noradrenergic fibres from NTS disinhibit histaminergic neurotransmission to the TMN, via α-2A-Rs. Furthermore, they showed that OEA recruited a subgroup of TMN neurons, as demonstrated by an increased number of c-Fos-positive nuclei, and increased the release of histamine from the cortex of 24-hour-fasted mice (Provensi *et al*, 2014). Because of the wide distribution of histaminergic projections in the CNS, it is reasonable that OEA indirectly increases histamine release in the PVN where activation of H1 receptors stimulates oxytocin release (Bealer and Crowley, 2001). Accordingly, Provensi and colleagues (2014) demonstrated that OEA-induced activation of oxytocic neurons in the PVN was blunted in histamine-deficient mice, an observation that could account for the inefficacy of OEA in these animals. The central histaminergic system seems to be involved also in the consummatory phase of feeding: as a matter of fact a gustatory information may also modulate the activation of the central histaminergic system physiologically, via taste nerves (Smith *et al*, 2012) and by emotions evoked by taste perception (Treesukosol *et al*, 2005). Evidence shows that only aversive taste stimuli actually increase the release of central histamine, not pleasant ones: these finding suggest the hypothesis that palatable food blunts histamine release resulting in overeating (Ishizuka *et al*, 2006). Not only taste perception, but also sensory information related to food texture can influence histaminergic activity. As an example (Ishizuka *et al*, 2006), histamine release increased from the amygdala when rats were fed hard pellets, while no significant differences were observed when the animals ate soft pellets. In our understanding, brain histamine serves as a relay station integrating peripheral signals and central functions, not only to control energy expenditure and appetite, but presumably also to influence the emotional value of different experiences (Benetti *et al*, 2015), such as the hedonic value of food (Provensi *et al*, 2015).

Histaminergic ligands in weight control: preclinical and clinical studies

From a therapeutic standpoint no brain penetrating H1 receptor agonists have been identified that would have anti-obesity effects. Infact, it will be challenging to identify H1 receptor agonists to be delivered selectively to the CNS without activating at peripheral H1 receptors leading to severe cardiovascular, respiratory, or gastrointestinal side effects. Therefore, the use of compounds that enhance histamine release form nerve terminals, such as H3 receptor antagonists/inverse agonist afforded an alternative strategy. Despite the encouraging preclinical results, though, clinical trials with H3 receptor antagonists for the treatment of obesity or feeding disorder were disappointing. For instance, a multicenter, randomized, placebo-controlled phase II clinical trial that evaluated the efficacy of the H3 receptor antagonist SCH497079 on weight loss in obese and overweight subjects was recently completed, but the results were not disclosed

(www.clinicatrials.gov). However, the consistent evidence for histamine being an anorexigenic agent, prompted researchers to change strategy and tested betahistine in obese patients. An early study showed that acute treatment of pigmy goats with betahistine, a structural analog of histamine with H1 receptor agonist and H3R antagonist properties, inhibited food intake and increased satiety (Rossi *et al*, 1999). In humans betahistine is used in the symptomatic treatment of vestibular disorders with a remarkable safety profile (Jeck-Thole and Wagner, 2006). It is orally available and readily penetrates the central nervous system. These properties encouraged clinicians to examine the effects of acute and chronic betahistine regimen in obese patients, as well as schizophrenics treated with atypical antipsychotics with propensity to induce weight gain (Barak *et al*, 2008). The acute effects of various doses of betahistine (48, 96 or 144 mg) on food intake and appetite were examined in a proof-of concept, randomized, placebo-controlled study in obese, otherwise healthy, women (BMI of 30–39.99 kg/m^2). Contrary to preclinical results, no significant effects of betahistine were observed in this cohort of obese women (Ali *et al*, 2010). Another study evaluated weight loss and other parameters (e.g. blood pressure) during a 12-week treatment period, in an obese multiethnic population. The study reported no significant weight loss with betahistine, however a post-hoc subgroup analysis revealed a significant effect on body weight with minimal adverse effects only in women below 50 years of age (Barak, 2008). Despite the disappointing results in obese, otherwise healthy patients, more promising results were reported in patients presenting a first episode of schizophrenia. Within the last 20 years, there has been a striking increase in the incidence of obesity and metabolic disorder in schizophrenic patients (Baptista *et al*, 2004) that seem to coincide with the development of a new class of neuroleptics with high affinity for the H1 receptor (Kroeze *et al*, 2003). Among these, olanzapine and clozapine that exhibit the highest binding affinities for the histamine H1 and muscarinic receptors (Teff and Kim, 2011) are associated with the greatest weight gain and metabolic impairments, including increased fasting glucose, insulin and triglycerides. Weizman and colleagues (Poyurovsky *et al*, 2005) were the first to report the beneficial effects of betahistine treatment in three patients hospitalized for a first episode of schizophrenic disorder. Betahistine at the dosage used to treat vertigo, was co-administered with olanzapine for six weeks. Although the lack of placebo controls preclude definitive conclusions, all patients after an initial weight gain during the first two weeks, had no additional increments, suggesting a stabilizing effect of betahistine. More recently, the same authors (Poyurovsky *et al*, 2013) used a combination treatment with reboxetine, a selective norepinephrine reuptake inhibitor, and betahistine to evaluate the olanzapine-induced weight gain in a small cohort of schizophrenic patients.

Compared to olanzapine/placebo-treated controls, patients in the combination therapy gained significantly less weight. It remains to be established if the combination reboxetine/betahistine offers a therapeutic advantage over betahistine alone. In this regard, the administration of betahistine in an animal model of olanzapine-induced weight gain was associated with decreased food intake and curbed weight gain (Deng *et al*, 2012). These results open the possibility that betahistine might exert weight-mitigating effects also in patients affected by other pathologies associated with obesity (e.g. diabetes mellitus) and reduce metabolic parameters relevant to weight gain. The promising effect of betahistine in preventing the metabolic side effects induced by atypical antipsychotic, but not in healthy obese patients may have to do with changes of the histaminergic system in the brain of people affected by schizophrenia, or plausibly, obesity. The level of tele-methylhistamine, the histamine metabolite that mirrors histamine release, is increased in the CSF of individuals with schizophrenia (Prell *et al*, 1997). A significant association between genetic variants of H1Rs (rs346074—rs346070) and BMI/obesity has been identified in non-affective, psychotic disorder patients treated with high H1 receptor affinity antipsychotics olanzapine, clozapine and quetiapine (Vehof *et al*, 2011). Post mortem studies found reduced H1 receptor binding in the frontal and prefrontal cortex and in the cingulate gyrus of individuals with schizophrenia (Iwabuchi *et al*, 2005), whereas H3 receptor binding, as measured by receptor radioligand binding autoradiography, was increased in the dorsolateral prefrontal cortex, but unchanged in the temporal cortex of patients with schizophrenia compared with the same brain regions in healthy control subjects (Jin *et al*, 2009). These differences, though, may reflect structural abnormalities of the cortical network and changes in cellular composition that underlie the functional impairments in this disorder. However, they may merely represent cytological adaptations in response to pharmacological treatment. It remains to be established if the morphological features of the histaminergic system in the brain of schizophrenic patients are responsible for the suggested beneficial effects of betahistine. Regarding obese patients, though, to our knowledge there are no published data that correlate modifications of the histaminergic system with weight gain or dysmetabolic pathologies. In light of these observations, several parameters were studies to understand the effect of betahistine in antipsychotic-induced metabolic disorders. Recent studies in rats showed that both subchronic (2 weeks; (Lian *et al*, 2014a)), and chronic (up to 4 weeks; (Lian *et al*, 2014b)) betahistine co-treatment prevented olanzapine-induced weight gain, feeding efficiency and fat mass. Furthermore, betahistine reverted several olanzapine-induced alterations, such as increased expression of hypothalamic H1 receptor, of pAMPK (a sensor of cellular energy status; (Shima *et al*, 2005)), and of the orexigenic

neuropeptide Y. Incidentally, the same authors reported in a previous paper that olanzapine subchronic or chronic treatment reduced H1R mRNA expression in the VMH and arcuate nucleus (ARC; (Han *et al*, 2008)). No explanations were provided to account for the discrepant results. Chronic olanzapine treatment also decreased the expression of UCP1 and PGC-1a (Stefanidis *et al*, 2009), two biomarkers of thermogenesis in the BAT, and again, this effect was prevented by co-treatment with betahistine (Lian *et al*, 2014b). Taken together these observations suggest that the mechanisms of betahistine in reducing olanzapine-induced weight gain and metabolic changes are through the modulation of the hypothalamic H1R-AMPK/BAT-UCP1-PGC-1a pathway. Presumably the central histaminergic information is conveyed via hypothalamic peptides secreted by the PVN or other hypothalamic nuclei such as the ARC, that signal to the BAT by activating sympathetic nerves (Masaki *et al*, 2004). Therefore, during treatment with second-generation antipsychotics, hypothalamic H1R antagonism not only increases appetite, but also reduces thermogenesis. Presumably, this is due to inhibition of the sympathetic outflows to the brainstem rostral raphe pallidus and rostral ventrolateral medulla, therefore decreasing BAT thermogenesis. Blocking of hypothalamic H_1 receptors may also contribute to fat accumulation by decreasing lipolysis and increasing lipogenesis in white adipose tissue. Furthermore, H1R blockade in the liver and pancreatic tissue will contribute to the onset of metabolic disorders (see for a review (He *et al*, 2013)). Betahistine is a weak H1R agonist and a more potent H3R antagonist that enhances histamine neuron activity (Gbahou *et al*, 2003) and histamine synthesis within the TMN (Lacour and Sterkers, 2001). Antagonists of the H3R decrease food intake in several mammalian species (Passani *et al*, 2011; Provensi *et al*, 2014). Therefore, the pharmacodynamic profile of betahistine may be responsible for preventing antipsychotic-induced metabolic side effects. As a weak H1R agonist, betahistine would compete with antipsychotics for binding to this same receptor, both centrally and in peripheral organs, whereas antagonism at the H3R would increase brain histamine release to curb appetite. Furthermore, the safety profile of betahistine indicates that this compound does not cause cardiovascular, respiratory, or gastrointestinal side effects.

AIM II

Provensi and co-workers recently showed in the mouse that a small population of histamine neurons responds to exogenous administration of OEA with increased activity, as demonstrated by increased *c-Fos* expression (Provensi *et al.,* 2014). Furthermore, we showed that histamine neurotransmission is necessary for OEA to promote hypophagia and to activate oxytocic neurons in the PVN. In the present study, we further study the hypophagic effect of OEA in the 24 hours after OEA i.p. administration and we explore the changes in neuronal activity induced by i.p. administration of OEA by assessing the pattern of *c-Fos* expression in brain regions that receive histaminergic innervation and that are involved in the control of feeding and emotional behaviours and may regulate the motivational aspect of foraging, in both mice deficient of the histamine-synthesizing enzyme histidine decarboxylase (HDC-KO), and their wild type (WT) littermates. Provensi et al., (2014) also described a diminished activation of the oxytocin-positive neurons in the PVN of HDC-KO mice compared to controls, in the present study we analyse neurohypophysis oxytocin immuno-density.

Materials and methods II

Animals

Housing, animals' maintenance and all experiments were conducted in accordance with the Council Directive of the European Community (2010/63/EU) of the Italian Decreto Legislativo 26 (13/03/2014), and National Institutes of Health guidelines on animal care and approved by veterinarian supervision. HDC-KO mice and WT littermates (129/Sv background) were grown in the animal facility of NEUROFARBA-Section of Pharmacology and Toxicology, Università di Firenze, housed in a temperature-controlled room (22 ± 1°C) with a 12:12-h light-dark cycle (light on 0700–1900 h), at a constant temperature and humidity with standard diet (4RF21; Mucedola s.r.l., Milan, Italy) and freely available water. HDC-KO and WT mice used at 2-3 months of age (25-30 g). Mice were handled for one week before experiments. Mice genotype was confirmed by PCR according to Provensi et al. (2014).

Detection of the Histidine Decarboxylase Gene

Mice genotype was confirmed by PCR according to Provensi et al. (2014). Inbred WT and histidine decarboxylase (HDC)-KO mice were used, a descendent from the 129/Sv mouse strain generated by Ohtsu et al. (2001). The genotype was determined by using the PCR protocol described by Parmentier et al. (2002). Briefly, the following primers were used for WT allele amplification: 5'-AGT GAG GGA CTG TGG CTC CAC GTC GAT GCT-3' (complementary to HDC gene 833–862) and 5'-TAC AGT CAA AGT GTA CCA TCA TCC ACT TGG-3' (HDC gene 980–951). The expected product size was 147 bp. The mutant allele was amplified using primers located within the Neor gene: 5'-AAA CAT CGC ATC GAG CGA GCA CGT ACT CGG-3' and 5'-ATG TCC TGA TAG CGG TCC GCC ACA CCC AGC-3', with an expected product size of 244 bp. PCR was performed using 40 cycles of 30 s at 94 °C, 1 min at 64 °C, and 1 min at 72 °C, followed by 1 cycle at 72 °C for 10 min. The whole reaction mix was then fractionated on a 2% agarose gel, and the PCR product was visualized by ethidium bromide staining.

Evaluation of food consumption

Mice were tested during the light-on period (starting at 9:00h) after 12h food-deprivation. Water remained available *ad libitum*. 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. Mice were randomly assigned to the experimental groups. Food consumption was measured 60 min, 2, 4, 6, 8, 10, 24 hrs after food presentation. OEA or vehicle were injected i.p. 15 min before food presentation.

c-Fos signle-staining

HDC-KO and WT littermates 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 vehicle. Mice were tested during light-on as in our previous study (Provensi et al., 2014). Two hours after OEA's injections mice were deeply anaesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with cold physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were post-fixed in the same solution overnight (4 ºC), and cryoprotected in 30% sucrose in PB. Forty µm thick sections were cut on a cryostat and collected in PB. Sections were preincubated in 0.75% H2O2 in PB for 30 min, in 0.2% BSA for 30 min and then incubated overnight in rabbit c-Fos primary antibody (1:5000; Sigma-Aldrich) at 4 ºC. The immunoreactive product was detected with the avidin-biotin peroxidase system (Vectastain kit; Vector Laboratories). After washing, sections were mounted on gelatin-coated slides, dehydrated, coverslipped and observed using an Olympus BX40 microscope equipped with a Nikon DS-F1 camera. c-Fos immunopositive nuclei were counted bilaterally using the Image J software (NIH, USA) on 3-4 sections per region/per mouse and normalized to a 1 mm2 area according to Munari et al. (2013). Atlas coordinates relative to bregma (Franklin and Paxinos, 2007) for the sections analyzed were from -1.34 to -1.58 for ARC, from -1.34 to -1.70 for BLA and CeA, from -1.46 to -1.94 for DMH, from -0.70 to -1.46 for LH and from-0.94 to -1.46 for SON. All regions analysed receive histaminergic fibers. Statistics were calculated on the average values from 3-4 sections of individual for each animal.

Oxytocin/*c-Fos* double-labelling

For double-labelling experiments, sections were preincubated with 2% normal goat and donkey sera (NDS, NGS Jackson Immunoresearch) for 1h and then incubated in a cocktail of c-Fos (1:3,000) and either mouse oxytocin (1:1,000; Millipore) primary antibodies with 1% NGS, 1% NDS overnight at 4 ºC. Sections were then incubated in Cy3-conjugated donkey anti-rabbit IgG (1:400; Jackson Immunoresearch) for 2h at room temperature and then in AlexaFluor 488-conjugated, goat anti-mouse IgG, (1:300; Molecular Probes) 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 (Provensi et al., 2014). Statistics were calculated on the average values of individual animals. Thus sample size and statistics were based on number of mice.

Oxytocin immunostaining of the neurohypophysis

The procedure was done according to Romano et al. (2013). Briefly, 18-µm-thick pituitary gland, sagittal sections were cut in a cryostat and mounted on gelatin-coated slides. Sections were incubated overnight at 4°C with anti-oxytocin antibody (1:1,000; Millipore), then incubated at room temperature for 2 h with anti-mouse Alexa Fluor 488 (1:1,000; Invitrogen) and mounted with anti-fading medium (Vectastain Vector Laboratories, Burlingame CA USA) containing DAPI to detect pituicyte nuclei. Sections were evaluated and photographed under an Olympus BX63 microscope equipped with a color charge-coupled device camera and controlled by the software NIS-Elements-BR (Nikon). In each pituitary gland section, we measured the optical density associated with Alexa Fluor 488 to semi-quantitatively evaluate oxytocin immunoreactivity. Statistics were calculated on the average values from 3-4 sections of individual for each animal.

Chemicals

OEA (Tocris Bioscience, Bristol, UK) was dissolved in saline/polyethylene glycol/Tween80 (90/5/5, v/v) and injected at 1 mL/100g of body weight. All other reagents and solvents were of HPLC grade or the highest grade available (Sigma, UK).

Data and statistical analysis

Statistical analysis was performed using Prism Software (GraphPad Software). Data are expressed as means ± SEM. Evaluation of cumulative food consumption was determined by two-way ANOVA followed by Bonferroni MTC. c-Fos expression in single-staining experiment was determined by one-way ANOVA with Tukey's post hoc test. Statistical significance between oxytocin immunopositive cells and double-labelled SON neurons in WT and HDC-KO mice were determined by unpaired t-test. Oxytocin optical density was determined by two-way ANOVA (genotype x treatment) with Newman–Keuls post hoc test.

Results II

The hypophagic factor oleoylethanolamide differentially induces an anorexiant effect and activates selective histaminergic pathways in the brain of histamine-deficient and normal mice

The integrity of the histaminergic system contributes to the acute anorexiant effect of lipid-derived molecule, oleoylethanomalide (OEA)

We evaluated the 24 hrs time course of the hypophagic effect of OEA in HDC KO mice and WT littermates. OEA was injected i.p. at 10 mg/Kg, whereas controls received equivalent volumes of vehicle. As previously shown (Provensi et al., 2014) OEA caused a significant reduction of the total amount of food consumed by WT mice compared to vehicle-treated littermates 60 minutes after injection ($F_{(time)6,108}$ = 162,96, P<0.0001; $F_{(treatment)3,108}$ = 3.70, p<0.05; $F_{(time\ x\ treatment)17,108}$ =0,52, ns ; two-way ANOVA followed by Bonferroni's test). A one-way ANOVA followed by Turkey's multiple comparison test, of food consumption at 60 min revealed a significant difference between groups: WT vehicle-treated animals consumed higher amounts of food compared to WT OEA-treated one, as well as HDC-KO vehicle treated compared to OEA treated ($F_{3,18}$ =16.31; P<0.001 and P<0.01, respectively) but the anorexiant effect of OEA in attenuated in HDC-KO mice compared to WT (P<0.05). Vehicle-treated HDC-KO mice consumed comparable amounts of food with respect to WT animals (P<0.001 at 60 min). Two hours after OEA injections, all experimental groups eat comparable amounts of food, independently of genotype and treatment (Figure II 1).

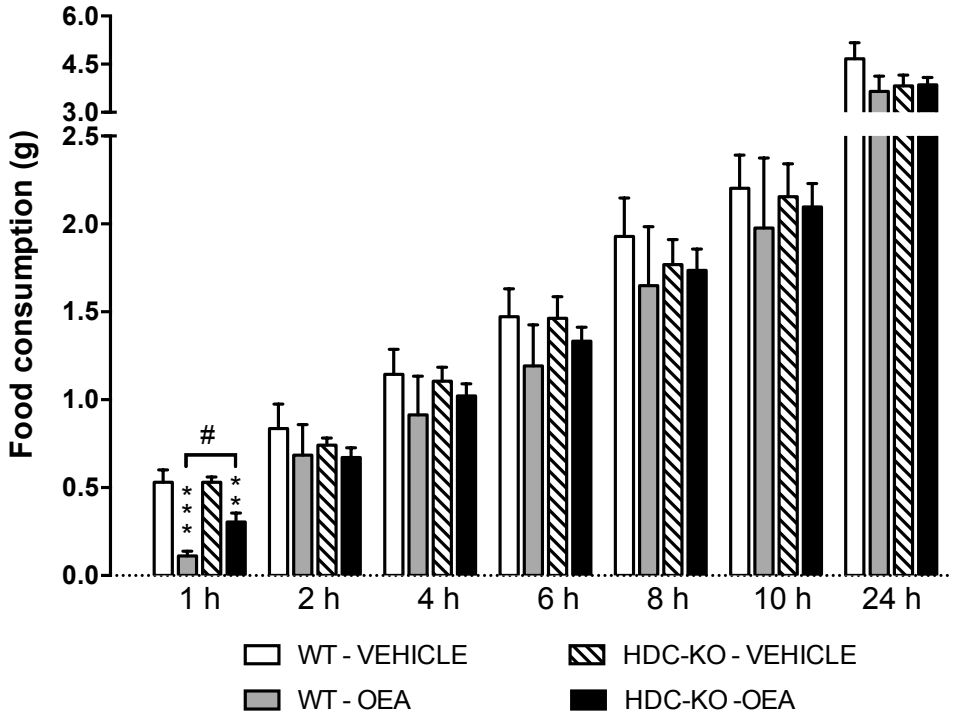

Figure II 1. Effect of oleoylethanolamide on food consumption in HDC-KO and WT mice.

Time course of cumulative food consumed by mice in the 24 hrs following OEA (10 mg/kg; i.p.) or vehicle administration. HDC-KO and WT mice were fasted for 12 hrs prior to treatments. Each point corresponds the mean ± SEM of 8-10 mice. ** P<0.01; *** P<0.001 vs. respective controls; $F_{(3,21)}$ = 16.31; two-way ANOVA followed by Bonferroni's MCT.

OEA differentially modulates *c-Fos* expression in the Supraoptic Nucleus (SON) and oxytocin density in the neurohypophysis of HDC-KO and WT mice

In this study we found that OEA treatment (10 mg/kg, i.p.) significantly increased c-Fos expression in the SON of WT mice compared to vehicle treated animals, ($F_{3, 44}$ = 9.638; P<0.01), while no differences were found in c-Fos expression in the SON of HDC-KO mice treated with vehicle or OEA (Figure II 2 A,B). The photomicrographs in panel C show oxitocyn positive cells (green) and c-Fos (red) in the SON of OEA-treated WT and HDC-KO mice, where apparently a higher number of double-labelled cells is found in WT mice.

OEA affected also oxytocin-immunoreactivity in the neurohypophysis as shown in Figure II 2D, E ($F_{3, 44}$ = 74.58; P<0.001). Post hoc analysis showed a significant increase of immunoreactivity in the neurohypophysis of OEA-treated WT mice, as compared to WT mice treated with vehicle. This effect however, was not observed in HDC-KO mice.

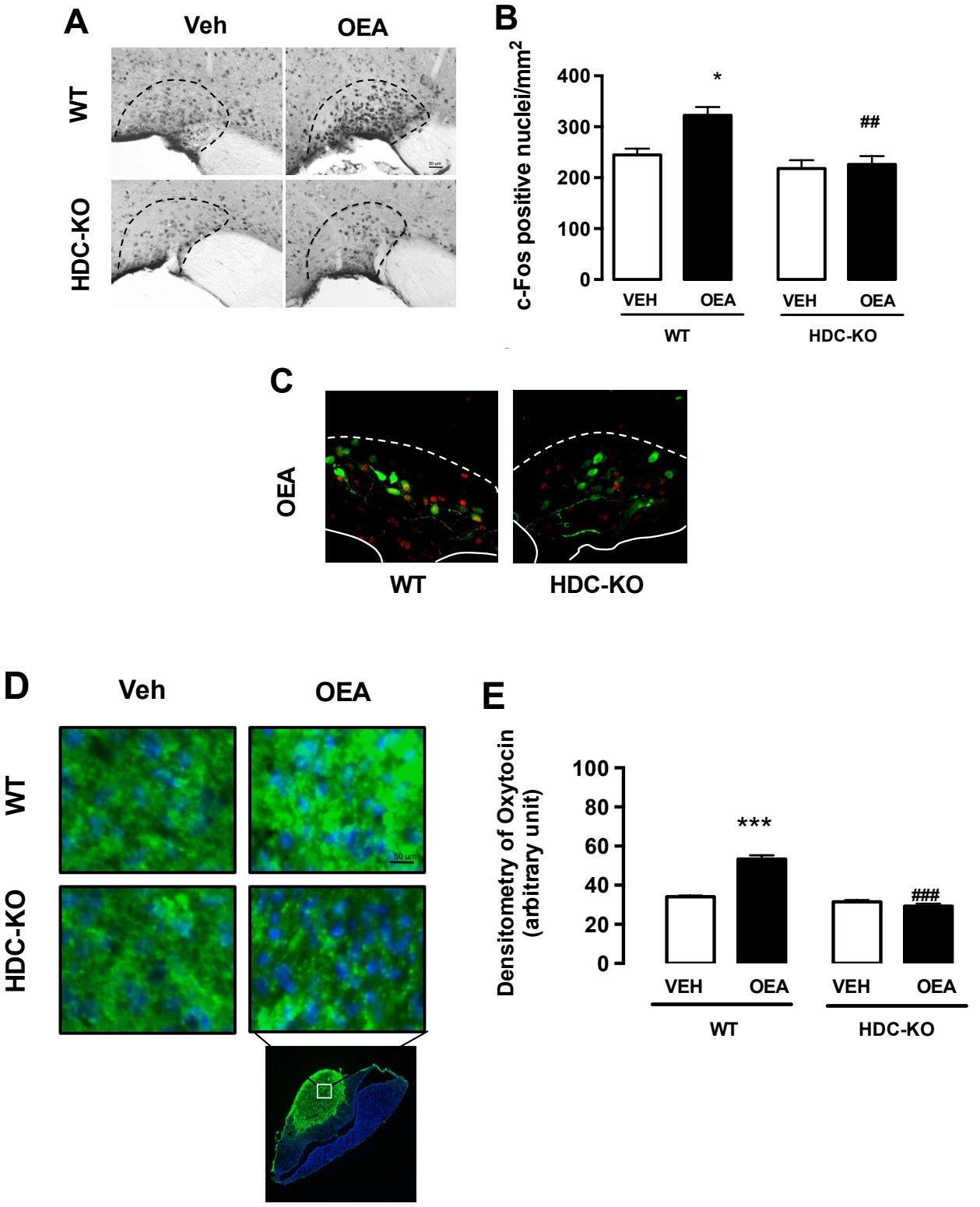

Figure II 2. Effect of oleoylethanolamide on c-Fos expression in the SON and oxytocin immune-density in the neurohypophysis of HDC-KO and WT mice.

Brain coronal sections show the effect of vehicle or OEA (10 mg/kg) on c-Fos expression in the SON of HDC-KO and WT mice (Fig. II 2A; Scale bar: 50 µm). Quantitative data are expressed as mean ± SEM of 4-5 mice for each experimental group. * P<0.05 VEH-treated WT vs. OEA-treated WT; ** P<0.01 OEA-treated WT vs. OEA-treated HDC-KO; one-way ANOVA followed by Turkey's MCT; Fig. II 2B. The photomicrographs in Figure II 2C show oxytocin positive cells (green) and c-Fos (red) in the SON of OEA-treated WT and HDC-KO mice. Immunohistochemical detection of oxytocin and DAPI (for nuclei) in the neurohypophysis of HDC-KO and WT-mice treated with OEA or vehicle (Fig. II 2D; Scale bar: 50 µm). Quantitative data are expressed as mean ± SEM of arbitrary unit of oxytocin immunodensity of 4-5 mice for each experimental group. *** P<0.001 vehicle-treated WT vs. OEA-treated WT; ### P<0.001 OEA-treated WT vs. OEA-treated HDC-KO; one-way ANOVA followed by Turkey's MCT; Fig. II 2E.

OEA differentially modulates *c-Fos* expression in brain regions related to feeding behaviour in HDC-KO and WT mice

In the arcuate nucleus (ARC) OEA augmented c-Fos expression significantly in WT mice ($F_{3, 18}$ = 17.56; p<0.0001; figure II 3A, B) with respect to vehicle treated mice, however, no difference were observed in HDC-KO mice treated with either OEA or vehicle. On the other hand, OEA did not affect c-Fos expression in the dorsomedial hypothalamusa (DMH) of either WT or HDC-KO mice compared to vehicle-treated mice ($F_{3,18}$ = 0.57; ns; Figure II 3C, D). Post hoc analysis did not reveal significant differences among experimental groups ($F_{3,15}$ = 0,71; ns; Figure II 3E, F). Similarly, neither genotype nor treatment differences were observed in the lateral hypothalamus

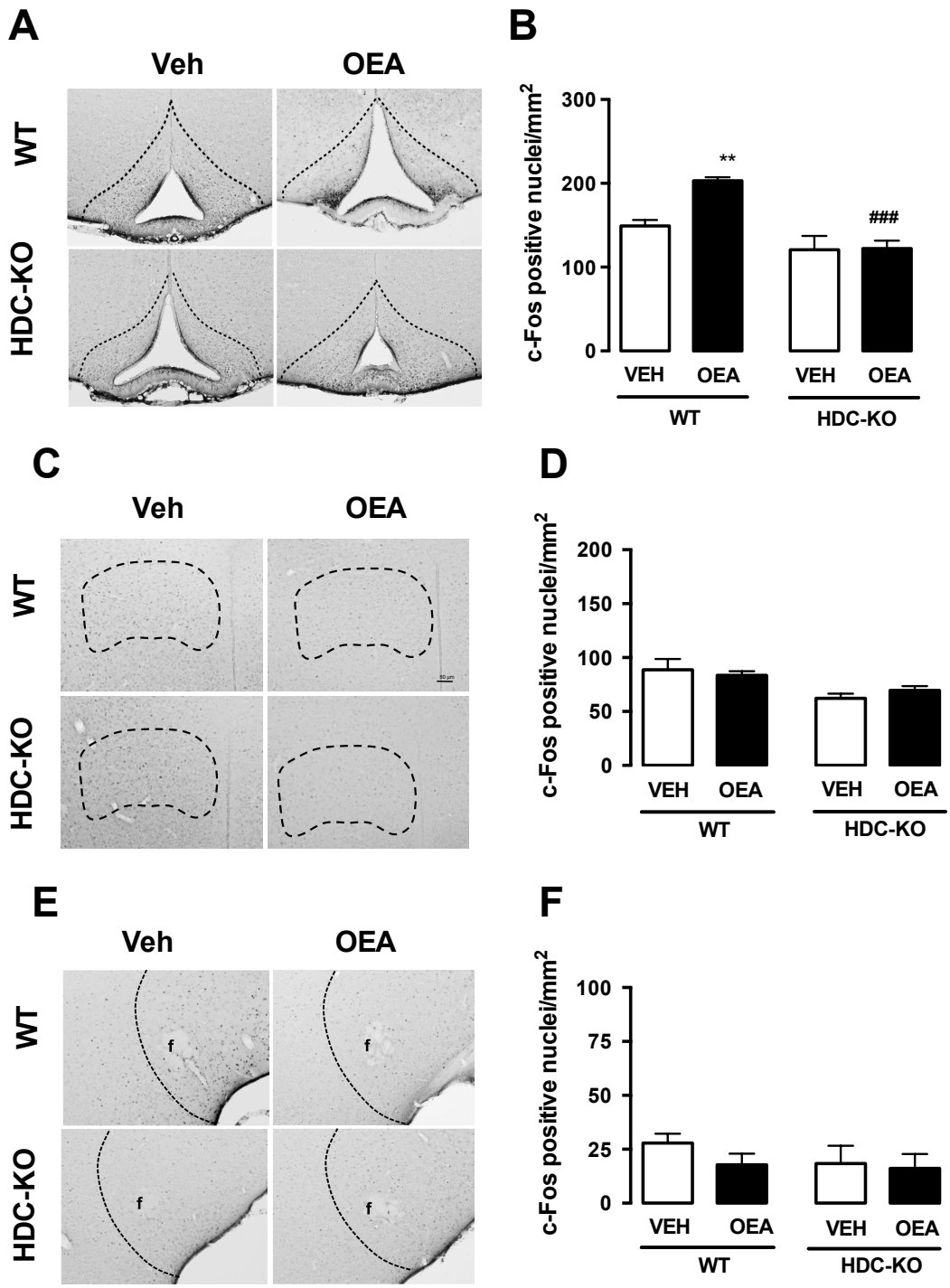

Figure II 3. Differential effect of oleoylethanolamide c-Fos expression in brain areas involved modulation of feeding behaviour in HDC-KO and WT mice.

Brain coronal section the effect of vehicle or OEA (10 mg/kg) on c-Fos expression in the ARC, DMH and LH of HDC-KO and WT mice (Fig. II 3; Scale bar: 50 µm). In panels A and B, quantitative data of ARC are expressed as mean ± SEM of 4-5 mice for each experimental group; ** $p<0.01$ vehicle-treated WT vs. OEA-treated WT; ### $p<0.001$ OEA-treated WT vs. OEA-treated HDC-KO; one-way ANOVA followed by Turkey's MCT. In panels C and D, quantitative data of DMH are expressed as mean ± SEM of 3-5 mice for each experimental group; ns; $F_{3,18} = 0.57$; one-way ANOVA followed by Turkey's MCT. In panels E and F, quantitative data of LH are expressed as mean ± SEM of 3-5 mice for each experimental group; ns; $F_{3,15} = 0,71$; one-way ANOVA followed by Turkey's MCT.

OEA differentially modulates c-Fos expression in the amygdala in HDC-KO and WT mice

We found that OEA treatment increased c-Fos expression in the amygdala of WT mice compared to vehicle treated animals. We examined both the central (CeA; $P<0.01$) and the basolateral (BLA; $P<0.001$) nuclei and observed comparable results (Figure II 3). In the CeA, a one-way ANOVA revealed a significant effect of treatment ($F_{3,11} = 10.73$; $P<0.001$; Figure II 3A, B) and post hoc analysis multiple comparison test revealed a significant difference between OEA- and vehicle-treated WT mice ($P<0.01$). This effect was not observed in HDC-KO mice. In the BLA, a one-way ANOVA and post hoc test revealed a significant effect of treatment ($F_{3,17} = 61.12$; $p<0.0001$; Figure II 3C, D), and a significant difference between OEA and vehicle-treated WT mice only.

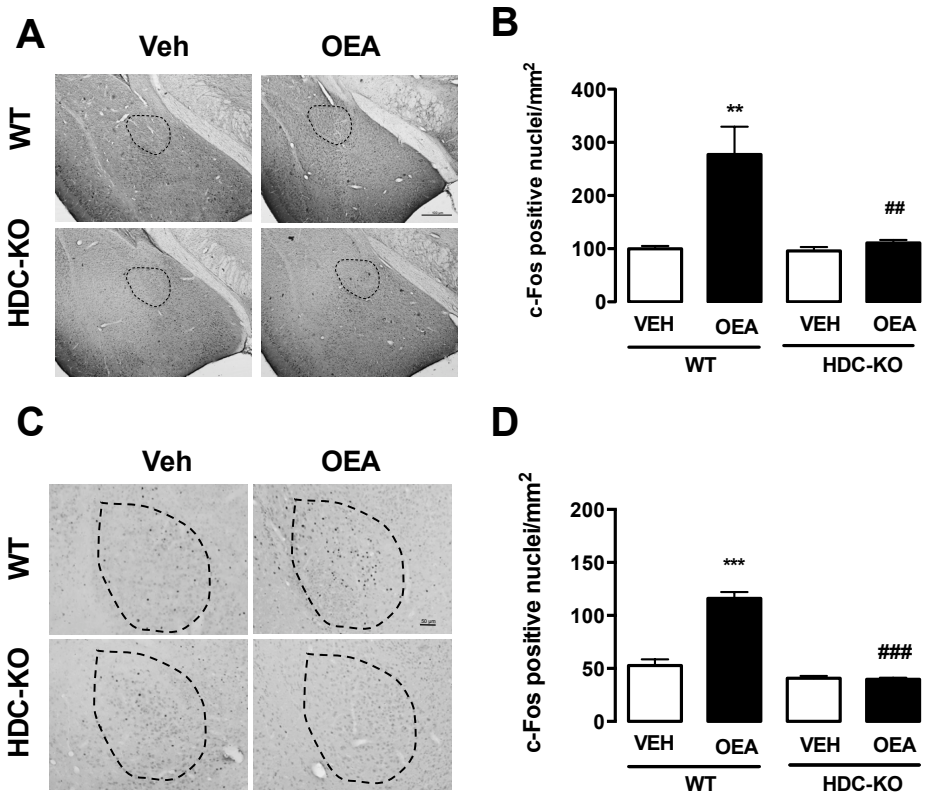

Figure II 4. Effect of oleoylethanolamide c-Fos expression in the amygdala of HDC-KO and WT mice.

Brain coronal section the effect of vehicle or OEA (10 mg/kg) on c-Fos expression in the CeA and BLA of HDC-KO and WT mice (Fig. II 4; Scale bar: 50 µm). In panels A and B, quantitative data of CeA are expressed as mean ± SEM of 4-5 mice for each experimental group; ** p<0.01 vehicle-treated WT vs. OEA-treated WT; ## p<0.01 OEA-treated WT vs. OEA-treated HDC-KO; one-way ANOVA followed by Turkey's MCT. In panels C and D, quantitative data of CeA are expressed as mean ± SEM of 4-5 mice for each experimental group; *** p<0.001 vehicle-treated WT vs. OEA-treated WT; ### p<0.001 OEA-treated WT vs. OEA-treated HDC-KO; one-way ANOVA followed by Turkey's MCT.

Conclusion II

The mechanisms for controlling eating behaviour involve the interplay between molecular signals secreted from the gut, the adipose tissue, neurohormones and central neurotransmitters. Food intake can be attenuated by multiple signals, including those associated with satiety, sickness and unpalatable tastants. The activity of histamine neurons that are clustered in the tuberomamillary nucleus (TMN) controls several homeostatic functions and behavioural responses such as sleep and aversive memory formation (Panula *et al*, 2013). Brain histamine seems to have different roles in different aspects of feeding behaviour, e.g. anticipatory vs consummatory phase (Valdez *et al*, 2007) and memory (e.g. consolidation vs. extinction, (Furini *et al*, 2014)). 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). Indeed, histamine neurons respond differently to endogenous (Sergeeva *et al*, 2005) and exogenous (Giannoni *et al*, 2009) molecules, and they show heterogeneous expression of histaminergic receptors (Blandina *et al*, 2012). The activity of histamine neurons is also regulated, directly or indirectly, by molecules generated in the gastrointestinal tract and by neurohormones such as leptin, corticotropin-, thyrotropin-releasing hormones and nesfatin-1 (reviewed in (Passani and Blandina, 2011; Provensi *et al*, 2015)). These promote satiety at least in part through histaminergic neurotransmission (Gotoh *et al*, 2013; Toftegaard *et al*, 2003). Oleoylethanolamide is a gut-derived satiety signal that inhibits eating and controls fat metabolism and energy expenditure, mainly through mechanisms involving activation of type- peroxisome proliferator activated receptors (PPAR- ; (Piomelli, 2013)). The anorexic effect of OEA results from the engagement of PPAR-α receptors in the gut and the recruitment of vagal afferents. The effect of OEA differs mechanistically from the serotoninergic anorexiant d-fenfluramine and the intestinal peptide CCK, mostly because OEA's effect strongly depends on feeding state of the subjects (Gaetani *et al*, 2003). As a matter of fact, i.p. administration of OEA (1-20 mg/kg) causes a dose-dependent delay in feeding onset in fed ad libitum animals without altering behavioural feeding patterns, whereas this substance evokes both delayed onset and reduced meal size in food-deprived rats (Gaetani *et al*, 2003).

The hypophagic effect is associated with the induction of c-Fos in brain regions that control food intake such as the nucleus of the solitary tract, the PVN and SON nuclei, the area postrema (Gaetani *et al*, 2010; Romano *et al*, 2013b). OEA stimulates oxytocin neurosecretion from the PVN (Romano *et al*, 2013a), and this hormone is involved in the regulation of homeostatic processes

and eating behaviour (Piomelli, 2013).

Activation of the H1R in CNS induces hypophagia in rats (Lecklin *et al*, 1998; Masaki *et al*, 2001), while the antagonism of this receptor increases meal size and duration. (Clinically, the importance of central histamine in the control of appetite is now accepted, as atypical antipsychotic drugs with high affinity for the histamine H1 receptor determine weight gain and dysmetabolic disorders (Deng *et al*, 2012; Kroeze *et al*, 2003; Provensi *et al*, 2015).

We recently demonstrated that the integrity of the central histamine system is necessary for OEA to suppress appetite and to activate oxytocic neurons in the PVN (Provensi *et al*, 2014). By using immunostaining for c-Fos we also demonstrated that a small population of histamine neurons responds to exogenous administration of OEA with increased activity (Provensi *et al*, 2014). Here, we further explore changes in neuronal activity induced by i.p. administration of OEA by assessing the pattern of c-Fos expression in brain regions that receive histaminergic innervation, in both mice deficient of the histamine-synthesizing enzyme histidine decarboxylase (HDC-KO), and their wild type (WT) littermates.

We extended our previous observations (Provensi *et al*, 2014) and compared the pattern of c-Fos expression induced by systemic administration of OEA in several brain regions of WT and HDC-KO mice innervated by the central histaminergic system and that are involved in the modulation of behavioural and emotional responses of eating. As a matter of fact here we confirm that hypophagic effect of OEA is attenuated in HDC-KO mice in a specific time frame (60 minutes after OEA administration) and coherently with BSS analysis (Provensi *et al*, 2014). We assume that increased c-Fos expression correlates well with increased activation of histaminergic afferents, as previously demonstrated by Munari (Munari *et al*, 2013). First of all, we found that OEA increased c-Fos expression in the SON and augmented oxytocin immuno-density in the neurohypophysis of fasted, WT mice, confirming the results obtained in rats fed *ad libitum (Romano et al, 2013a)*. All these effects were absent in fasted HDC-KO mice. We previously showed that OEA used at the same concentration as here, induces hypophagia in food restricted mice that have an intact histaminergic system (Provensi *et al*, 2014). These and our present results strongly suggest that OEA induces anorexia, at least in part, by indirectly stimulating histamine neurons in the TMN that project to the PVN and SON. The hypothalamus is an essential station in controlling feeding: insulin, via activating its receptors, induces phosphatidylinositol-3-kinase (PI3K) action that stimulates Akt (protein kinase B) leading to the expression of nuclear factors that decrease of transcription of the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) and increase the transcription of the anorexigenic factor pro-opiomelanocortin (POMC) with

consequent activation of the PVN and a reduction of food intake (Condes Areias and Oliveira Prada, 2015). In the hypothalamus OEA increased c-Fos expression in a small number of cells of the ARC of WT mice only. The ARC plays an important role in the regulation of appetite and energy homeostasis (Page *et al*, 2013). Here, we did not attempt to identify the cell types that express c-Fos. In rats, TMN projections to the ARC are activated in response to food-deprivation under a restricted feeding schedule (Umehara *et al*., 2012). The same authors (Umehara *et al*., 2010) also observed that c-Fos is significantly induced by food deprivation under scheduled feeding in H_1 receptor-expressing cells in the caudal part of the ARC, but not in the PVN or ventromedial hypothalamus. On the other hand, overnight starvation, as in our protocol, did not induce c-Fos in the ARC. Presumably, different histaminergic projections to the ARC are activated in different circumstance and homeostatic state of the animal, such as during OEA-induced satiation and food anticipation.

LH-lesioned rats have the ability to move, to eat and drink but loose all the motivation to do so: as a result, in Anand and Brobeck (1951) experiments, rats died for self-inflicted starvation and dehydration. By contrast, electrical stimulation of the LH promotes feeding and drinking behaviours, as well as increases physical activity (Delgado and Anand, 1953). The LH contains neurons expressing orexin, a neuropeptide that regulates sleep/wakefulness states and feeding behaviour (Sakura, 2014) and melatonin-concentrating hormone that is known to regulate a wide variety of physiological functions such as feeding, metabolism, anxiety, depression and reward. Thus, the LH in not just a “feeding centre” and must be considered in terms of how it coordinates a spectrum of ingestive and arousal behaviours relevant to energy expenditure (Brown et al., 2015). The histaminergic and orexinergic systems are reciprocally connected in an excitatory fashion (Anaclet et al., 2009; Schöne et al., 2012), whereas histamine strongly inhibits MCH neurons via activation of H_3 receptors (Parks *et al*., 2014). OEA apparently did not affect these circuits, as no OEA-induced changes in c-Fos expression were found in the lateral hypothalamus of either genotype. Soria-Gómez et al. (2010) reported that systemic OEA induced a significant reduction of c-Fos positive cells in the LH of rats fed ad libitum. Presumably, the different species used and/or the homeostatic states of the animals (fasted vs. fed) are responsible for the different patterns of c-Fos expression.

Even the dorsomedial hypothalamic is fundamental in feeding behaviour control but, as in the lateral hypothalamus, it strongly depends on the arousal state: as an example, NPY neurons in DMH are strongly activated and increase food consumption in response to chronic food restriction but not in acute food deprivation (Bi, 2007). Coherently, OEA administration did not induce c-Fos

expression differences in either genotypes in the DMH, which is part of an intra-hypothalamic system controlling food anticipatory behaviour (Acosta Galvan *et al*., 2011).

Bilateral amygdaloid lesions induced hyperphagia and weight gain (King *et al*., 1994) and hyperinsulinemia (King et al., 1996) in female rats. Interestingly, OEA increased c-Fos expression in the amygdala, both in the CeA and BLA. CeA neurons receive presynaptic inputs from anatomically distributed neurons activated by different anorexigenic agents (Cai *et al*., 2014) and c-Fos expression in this nucleus is stimulated by a range of anorectic stimuli including GLP-1 and glucagone (Baumgarten *et al*., 2010; Parker et al., 2013). Furthermore, intra-CeA infusion of anorexic compounds, including those regulating the melanocortin system or opioid signaling, affect food intake when infused into this brain region (Kask *et al*., 2000; Beckman *et al*., 2009; Fekete *et al*., 2002; Fekete *et al*., 2007; Kovács *et al*., 2012). Hence, our data suggest that the CeA has an important part in mediating the anorectic effects of OEA, which is consistent with the strong activation seen in this nucleus. Furthermore, here we demonstrate that OEA-induced activation of CeA requires the histaminergic neurotransmission.

The significance of OEA-induced BLA activation is less clear. It was recently shown that OEA facilitates aversive memory consolidation through noradrenergic activation of the BLA (Campolongo *et al*., 2009), and preliminary data from our laboratory indicate that this facilitation is lost in histamine-deprived rats (Provensi *et al*., 2015). The BLA is also critical for flexible, goal directed behaviours, such as the devaluation of a reinforcer like palatable food (West *et al*., 2012). Whether the BLA also participate in OEA-induced regulation of feeding behaviour is worth exploring by using specific behavioural paradigm and pharmacological manipulations.

In conclusion, we described the expression of c-Fos induced by OEA in previously unexplored brain areas. Differences between other reports (e.g. Gaetani *et al*., 2010) may be attributable to the use of satiated vs well-fed animal, the measurement of c-Fos mRNA vs the protein and the time of the day when the experiments were performed (light on/light off). In this regard, we previously showed that the anorexic effect of OEA is almost completely abolished in histamine-deprived mice when food consumption is measured at dark onset, when the animals are naturally more aroused and motivated to eat (Provensi *et al*., 2014). On the other hand, when we used the same protocol during the light period, the hypophagic effect of OEA was partially blunted in histamine deprived-animals. Hence, histamine is presumably released in a 'state dependent' manner.

The results presented here provide an indication that OEA and brain histamine interact only in selective hypothalamic regions to control feeding behaviour in a paradigm where food is made available after a 12-hour restriction. Hence, our data suggest the existence of a complex

histaminergic circuit modulated by endogenous signals such as OEA that activate TMN neurons in a selective fashion according to their projection area and these systems control in concert homeostatic and motivated behavioural responses.

We believe that understanding the role of the histaminergic system in driving or modulating feeding behaviour is of relevance for the development of more effective pharmacotherapy for the management of eating disorders and the improvement of the safety profile of centrally acting drugs.

CHAPTER IV

BRAIN HISTAMINE IN THE MODULATION OF OEA PRO-MNESIC EFFECT

The Basolateral Amygdala as a centre for storing emotion and memory: an optogenetic point of view

Perhaps the oldest line of research on the amygdala's role in the brain originates with how the amygdala regulates emotion and emotional output. Indeed, early work showed that amygdala lesions in monkeys produced the now-classic Kluver-Bucy syndrome (WEISKRANTZ, 1956). The symptoms of the syndrome involved profound alterations in the monkeys' emotional behaviour, especially those involving fear-based behaviour. These early findings have led to a considerable number of studies that have expounded upon this function for the amygdala. Studies in humans have confirmed the findings on amygdala lesions, as selective amygdala lesions also appear to produce deficits in emotion-related behaviours, especially those regarding fear (Adolphs *et al*, 2005).

Thus, it is not surprising that studies have harnessed the power of optogenetics to develop an improved understanding of how the amygdala influences emotional behaviour, as shown, for instance by Tye et al. (Krueger *et al*, 2011) who unravels the function of amygdala in anxiety and anxiety-related behaviours. Tye et al. used an optogenetic approach to distinguish the roles of the basolateral amygdala (BLA) inputs to the medial vs. lateral portions of the central amygdala (CEA). Indeed, previous work had suggested that the medial CEA and its inputs from the BLA drive anxiety and/or fear-related behaviours, whereas BLA inputs to the lateral CEA provide feed-forward inhibition of the medial CEA (Meins *et al*, 2010). To distinguish these pathways, Tye et al. (2011) transduced BLA neurons with either the depolarizing cation channel channelrhodopsin-2 (ChR2) or the hyperpolarizing chloride pump halorhodopsin and illumination was provided to the BLA terminals in the lateral CEA. The expression of the opsins was limited to the pyramidal neurons of the BLA through the use of a CaMKIIα promoter. The results indicated that stimulation and inhibition of the BLA terminals in the lateral CEA reduced and increased, respectively, anxiety in the mice, as measured in different tasks. Moreover, the findings strongly suggested that this effect was due to feed forward inhibition of the medial CEA. Illumination of the BLA cells themselves did not produce the same behavioural effect and sometimes produced the opposite effect. With low light levels, illumination of the terminals did not produce reliable antidromic propagation of action potentials back to the BLA, largely eliminating the concern that such propagation could be responsible for the behavioural effect, although optical stimulation of axon terminals can produce reliable antidromic propagation to cell bodies (Jennings and Ordonez, 2013). These results not only provided a clearer understanding of the amygdala circuits driving

behaviour but illustrated the importance of targeting specific downstream projection areas, as stimulating or inhibiting specific projections may produce rather different effects.

Since this initial work, Tye et al. have extended their focus on anxiety by examining the projections from the BLA to the ventral hippocampus in the regulation of anxiety-related behaviours (Felix-Ortiz et al., 2013). Previous studies had indicated that the BLA projects to the ventral hippocampus and that the ventral hippocampus is also involved in anxiety (Pitkänen et al., 1995, 2000; Bannerman et al., 2003). Using an open-arm plus maze and an open-field chamber, Felix-Ortiz et al. found that inhibition of BLA afferents to the ventral hippocampus, via activation of halorhodopsin, decreased anxiety-related behaviours. Similarly, stimulation of such afferents, using 20 Hz light pulses to activate ChR2, increased anxiety-related behaviours. Importantly, control experiments demonstrated that the effects of BLA terminals stimulation were not due to antidromic stimulation of the BLA itself or orthodromic stimulation of fibres of passage. Moreover, electrophysiology analysis suggested that the effects of stimulating BLA inputs to the ventral hippocampus were mediated through local circuit mechanisms involving both direct activation of principal cells in the hippocampus and indirect recruitment of inhibitory neurons. In another work, Felix-Ortiz and Tye (2014) examined optical stimulation and inhibition of the BLA axon terminals in the ventral hippocampus while testing mice in a social behaviour. In a resident-juvenile-intruder test, stimulation of these terminals, using 20 Hz light pulses, reduced social interactions of the resident, whereas inhibition of these terminals increased such interactions, suggesting that activation of this pathway increased anxiety in the animals whereas silencing of this pathway produced the opposite effect. The effects with stimulation were blocked with microinjections of glutamate receptor antagonists into the ventral hippocampus, indicating that the results were due to stimulation of BLA axon terminals and the concomitant release of glutamate from those terminals. Similar behavioural effects with optogenetic stimulation were observed with a three-chamber sociability test. Together, the studies by Tye et al. have produced a wealth of knowledge regarding how the amygdala, and especially the BLA, influences anxiety-related behaviours through different outputs to other regions.

Other work has focused on how inputs from the hypothalamus regulate amygdala activity and fear-related behaviours. A study indicated that oxytocin exerts its effects on a variety of behaviours, at least in part, through activation of oxytocin receptors in the CEA (Viviani et al., 2011). However, while oxytocin neurons are located in the hypothalamus, it has not clear whether oxytocin is released via dendritic mechanisms and then spreads passively to the amygdala or it is released via classic axonal mechanisms (Landgraf and Neumann, 2004). To address this issue,

Knobloch et al. (2012) transduced hypothalamic oxytocin neurons using an oxytocin promoter. Optical stimulation of these neurons' axonal terminals in the CEA produced oxytocin-dependent effects on CEA neuronal activity, indicating that the hypothalamic neurons are able to directly release oxytocin from their axon terminals. In particular, optical stimulation appeared to increase neuronal activity in the lateral CEA and inhibit activity in medial CEA neurons. Anatomical analysis found that the hypothalamic inputs terminated in the lateral, but not medial, CEA. A separate experiment providing stimulation during a test for contextual fear conditioning found an attenuation of freezing behaviour with the optical stimulation. Indeed, the overall evidence from this work suggests that the hypothalamic oxytocin neuronal input to the CEA makes synaptic contacts on the lateral, rather than medial, aspect of the CEA, consistent with the function of the lateral CEA in inhibiting medial CEA output and reducing the expression of fear and fear-related behaviour. On a larger level, these findings contribute to our understanding of how specific inputs, and even genetically distinct inputs, to the amygdala regulate both behaviour and local circuit activity.

Amygdala and memory

Other work has focused on the use of optogenetics to understand the relationship between the amygdala and memory, especially aversive learning such as fear conditioning. One of the earliest uses of optogenetics in studies of the amygdala examined whether optogenetic stimulation of the lateral amygdala, combined with tones, produces fear conditioning (Johansen et al., 2010). In these experiments, the authors targeted the pyramidal cells of the lateral amygdala. These early findings confirmed that different frequencies of light produced robust firing in lateral amygdala neurons. With 20 Hz stimulation, there was a strong c-fos response in the neurons. Optical stimulation, paired with a tone, produced an increase in freezing both during training and in a later retention test. However, the authors noted that the levels of freezing were considerably lower than had been found in previous studies, an effect that the authors suggest indicates that other mechanisms must also be involved in order to produce full fear conditioning. These findings contributed to previous work showing that activity in the lateral amygdala is critical for the normal development and retention of tone fear conditioning. Moreover, through the use of optogenetics, these experiments were able to selectively target the pyramidal cells while providing temporally precise stimulation.

Huff and colleagues used an optogenetic approach to examining the role of the BLA in modulating memory consolidation for inhibitory avoidance, a similar aversive learning task (Huff et al., 2013).

Prior work suggested that BLA activity in the gamma frequency range (35–45 Hz) is important for synchronizing activity in downstream structures and promoting the consolidation of learning (Bauer et al., 2007; Popescu et al., 2009). However, such work has depended on physiological recordings, which cannot determine whether driving activity in that range alters memory consolidation. Huff and coworkers found that stimulating the BLA pyramidal cells with bursts of gamma-frequency light pulses (40 Hz) for 15 min immediately after inhibitory avoidance training enhanced retention 2 days later (Huff et al., 2013). Stimulation with bursts of 20 Hz pulses did not produce a significant effect on retention. Previous work has shown that other types of post-training stimulation (e.g., electrical) of the amygdala produce an inverted-U curve with regard to retention (Gold et al., 1975), but it is not known whether optical stimulation of the BLA also produces such an effect. In another groups of rats, Huff inhibited BLA neuronal activity immediately after training via activation of the outward proton pump ArchT and found that 15 min of neuronal inhibition, but not 1 min, impaired retention of the learning (Huff et al., 2013). These findings indicated that BLA stimulation in the gamma frequency range enhances memory consolidation.

Other work has investigated a subpopulation of BLA neurons to examine its role in fear conditioning (Jasnow et al., 2013). Specifically, Jasnow et al. targeted the glutamatergic pyramidal cells found in the BLA by driving ChR2 expression with the Thy1 promoter, which limits expression to a specific subpopulation of glutamatergic cells in the BLA and other forebrain regions (Sugino et al., 2006). Optical stimulation of this specific class of BLA neurons during tone fear conditioning impaired retention, while having no effect on the expression of freezing itself during the conditioning (Jasnow et al., 2013). Moreover, optical stimulation of the neurons paired with the tone alone during extinction training enhanced the retention of the extinction learning, again without having any effect on the freezing itself during the extinction training. Generally, it has been thought that activity in the lateral amygdala and medial CEA drive the expression of fear, but electrophysiological characterization of this subpopulation suggests that it shunts activity in lateral amygdala neurons and inhibits activity of medial CEA neurons. Indeed, optical activation of this neuronal subpopulation had no effect on the acute expression of fear but, rather, appeared to influence consolidation specifically for memories that *oppose* fear conditioning.

Recent work has also investigated the interactions of distinct subpopulations within the BLA with efferent targets in relation to fear conditioning. Prior work had found the existence of neurons within the basal nucleus of the BLA that are responsive to cues associated with footshocks and other neurons that are responsive to cues previously associated with footshocks that have been

extinguished. Senn et al. (2014) investigated whether such neurons also show distinct projection patterns to the medial prefrontal cortex (mPFC). To perform a functional investigation of these subpopulations of neurons, retrogradely transported viruses containing Cre-recombinase were injected into either the prelimbic (PL) or infralimbic (IL) cortex while a conditional viral vector expressing the opsins in a Cre-dependent manner was injected into the BLA. As a result, the opsins were selectively expressed in the IL-projecting neurons or the PL-projecting neurons, enabling illumination of the entire BLA to only stimulate or inhibit the specific subpopulation of neurons. Consistent with previous work suggesting a dichotomy between the dorsal regions of the mPFC (PL) and the ventral regions (IL) with regard to fear conditioning (Peters et al., 2009), the authors demonstrated that BLA neurons projecting to the PL are activated by unextinguished cues whereas those projecting to the IL are activated by extinguished cues. Moreover, inhibition of IL-projecting neurons during cue extinction training produced a significant impairment in the retention of the extinction learning, compared to stimulating such neurons. Conversely, inhibition of the PL-projecting neurons during such training enhanced the retention of the extinction learning, compared to stimulating such neurons. While much of previous research had focused on the mPFC inputs to the BLA in regulating fear extinction and expression, these findings provide evidence that BLA inputs to distinct mPFC regions also differentially influence such behaviours.

Circuitry of the amygdala and interactions with other brain regions

A number of studies have used optogenetic approaches to develop a better understanding of the functional connections between the amygdala and other brain regions. For example, Li et al. (2012) have investigated the role of kappa opioid receptor signaling in the bed nucleus of the stria terminalis (BNST). Patch-clamp recordings in the BNST provided evidence that activation of kappa opioid receptors inhibits GABAergic transmission via presynaptic mechanisms. As the CEA provides an important GABAergic input to the BNST, one that has been implicated as a critical pathway in the central stress system (Jasnow et al., 2004; Walker and Davis, 2008), the authors used optogenetics to target and control activity in this pathway. The results indicated that kappa opioid receptor activation inhibited GABAergic transmission in this pathway specifically, a result that would have been difficult to demonstrate using other techniques.

Other research has used optogenetics to delineate precisely how the amygdala influences activity in other regions. Luna and Morozov (2012) blocked the microcircuitry of BLA inputs vs. anterior piriform inputs to the posterior piriform cortex. Although both structures were found to innervate deep pyramidal cells of the posterior piriform, the BLA and anterior piriform connected with different kinds of interneurons. Specifically, the BLA produced strong connections with fast-spiking

interneurons, whereas the anterior piriform had its strongest synapses on irregular-spiking interneurons. As these different classes of interneurons synapse on different regions of the pyramidal cells (somatic vs. distal dendritic), the feedforward inhibition from BLA vs. anterior piriform inputs would be expected to have profoundly different effects on the likelihood of spiking in the principal cells of the posterior piriform.

Several studies have used optogenetics to understand amygdala function and interactions in combination with a variety of other techniques. For example, experiments have focused on the well-known connections between the BLA and the mPFC. The reciprocal connections between these regions appear to be involved in a wide variety of behavioral and higher cognitive functions. Yet, the mPFC receives inputs from many other structures and the distinctions among the connections formed by these inputs have not been clear. Therefore, Little and Carter (2012) investigated how BLA, ventral hippocampal, midline thalamus, and contralateral mPFC inputs to the layer II pyramidal neurons of the mPFC make synaptic connections. The authors used optogenetics to target specific pathways by transducing the efferent structures with ChR2 and providing illumination to their axonal terminals in the mPFC. Moreover, they combined their optogenetic manipulations with two-photon microscopy in order to determine the functional connections on a subcellular level. The results from this study indicate that the different regions do, in fact, make different subcellular connections. The BLA appears to make synaptic connections much closer to the soma, relative to other regions, especially the thalamic inputs. Additionally, the BLA inputs target spines of an “intermediate” size, along with ventral hippocampal inputs, in contrast to the thalamic inputs to the large spines and the contralateral mPFC inputs to the small spines. As both the size of the spine and the distance from the soma govern the relative strengths of the inputs, these findings shed light on how different regions influence local circuit activity in other regions. In a follow-up study, Little and Carter (2013) extended their findings, again using optogenetics combined with two-photon microscopy. In this case, their findings indicated that BLA inputs to the mPFC were considerably stronger on mPFC neurons that innervated the BLA, compared to mPFC neurons that provide inputs to the contralateral mPFC. Together, these findings have contributed to a deeper understanding for how the BLA and mPFC interact and, critically, provide a foundation for understanding how BLA inputs to the mPFC may regulate PFC activity and PFC-dependent functions.

Other works examined other brain regions’ inputs to the amygdala. In a recent study, Carter et al. (2013) found evidence of a circuit involving projections from the parabrachial nucleus in the brainstem to the CEA that suppresses appetite. After genetically identifying and targeting neurons

in the parabrachial nucleus to determine their ability to suppress appetite in mice, the authors then transduced these cells with ChR2 and provided illumination to downstream targets. Although stimulating parabrachial axon terminals in the BNST had no effect on food intake, stimulation between 20–40 Hz of the axon terminals in the lateral CEA reduced food consumption. By utilizing both the genetic targeting ability of combining optogenetics with transgenic mice and by targeting the axon terminals, these findings provide a significant step forward in understanding how genetically distinct neuronal populations connect with different regions in the brain and, in turn, regulate appetite-related behavior.

Optogenetic studies have also targeted specific interneuronal populations in the BLA to understand local circuits. Chu et al. (2012) examined how dopamine influences parvalbumin-positive interneurons in the BLA and, thereby, influence principal cell activity. This issue is of importance because previous work has shown that dopamine influences BLA activity and modulates memory consolidation (Bissiere et al., 2003; LaLumiere et al., 2004, 2005). Prior work has found that D2 receptor activation in the BLA suppresses feedforward inhibition, thereby providing a gating mechanism for synaptic plasticity in the amygdala (Bissiere et al., 2003), while other work has also found that dopamine disinhibits the BLA via inhibition of intercalated cells in a D1 receptor-dependent manner (Marowsky et al., 2005). Using a Cre line of transgenic mice, Chu et al. were able to target ChR2 expression to the parvalbumin-positive cells of the BLA, which are believed to be the major class of interneurons in the structure. The authors then demonstrated that dopamine selectively reduced GABAergic transmission to principal cells, but not to other interneurons, and that this occurred in a presynaptic D2 receptor-dependent mechanism. These findings provide additional confirmation of the critical role of dopamine, and especially D2 receptors, in modulating BLA activity. Moreover, these findings demonstrated in a specific subclass of interneurons, an important issue as other work has suggested that different stimuli influence different subtypes of interneurons in the BLA (Bienvenu et al., 2012).

The lipid-derived satiety factor, oleoylethanolamide, in memory consolidation

Food ingestion stimulates enterocytes in the vertebrate small intestine to produce the fatty-acid amide oleoylethanolamide (OEA) (Rodriguez de Fonseca et al., 2001; Fu et al., 2007; Astarita et al., 2006), a potent endogenous agonist of type-α peroxisome proliferator-activated receptors (PPAR-α; Fu et al., 2003). Acting as a local messenger within the gut (Fu et al., 2008), newly formed OEA stimulates enterocytes to express PPAR-α–regulated genes involved in lipid absorption, such as

fatty-acid transporters and binding proteins (Fu et al., 2003; Yang et al., 2007), and engages afferent fibers of the vagus nerve to delay further eating (Rofriguez de Fonseca et al., 2001; Oveisi et al., 2004; Schwartz et al., 2008).

The ability to remember important contextual information about food sources, including their exact location and safety of access, is clearly advantageous to animals foraging in the wild (Roberts et al., 2008; Zinkivskay et al., 2009). It is also well established that stress hormones activated by emotional arousal enhance memories of cues associated with the arousal (McGaugh and Roozendaal, 2002; McGaugh, 2002). Emotional arousal facilitates the consolidation of memory traces, an adaptive phenomenon that is primarily mediated by secretion of the adrenal stress hormones, epinephrine and cortisol, into the bloodstream (McGaugh and Roozendaal, 2002; McGaugh, 2002). Circulating epinephrine does not enter the brain and is thought to initiate its memory-enhancing effects by activating β-adrenergic receptors located on sensory terminals of the vagus nerve. The afferent signal generated by epinephrine projects to the NTS in the brainstem, where it stimulates noradrenergic neurons that activate the BLA and other forebrain structures (McGaugh, 2004). Norepinephrine release in the BLA is particularly critical for mediating the effects of peripheral epinephrine on memory consolidation; indeed, infusions of β-adrenergic receptor antagonists into the BLA block such effects, whereas infusions of β-adrenergic receptor agonists mimic them (McGaugh, 2000; McGaugh, 2004; Gonales-Yanes et al., 2005).

Campolongo and colleagues (2009) reveal striking mechanistic similarities between the memory-enhancing actions of epinephrine and those of OEA. Like epinephrine (McGaugh, 2004), OEA increases memory consolidation by eliciting an autonomic signal that reaches the forebrain through the NTS and results in the noradrenergic activation of neurons in the BLA. Accordingly, infusions of the local anesthetic lidocaine into the NTS or the β-adrenergic antagonist propranolol into the BLA prevent the memory-enhancing effects of both epinephrine (McGaugh, 2000) and OEA. The two hormones are markedly different, however, with respect to the physiological context in which they operate. Epinephrine is released from the adrenal gland during arousal and stress, whereas OEA is produced by small-intestinal enterocytes in response to the arrival of dietary fat (Roberts et al., 2008). This suggests that salient stimuli of diverse modalities—nutritional as well as emotional—converge on the same neuromodulatory system in the brain to facilitate memory consolidation.

Despite these mechanistic commonalities, epinephrine and OEA have profoundly different consequences on behaviour. Epinephrine administration can produce a behavioural state characterized by heightened anxiety and incidence of panic attacks (Veltman and Gaillard, 1998).

By contrast, even at doses that maximally inhibit food intake and enhance memory, OEA does not change rats' behaviour in a novel open field and does evoke anxiety-like responses in the elevated plus-maze. Moreover, OEA does not increase plasma glucose levels, a typical effect of adrenergic activation, and does not evoke corticosterone release (Rodriguez de Fonseca, 2001; Gonzalez-Yanes et al., 2005). This profile distinguishes OEA not only from epinephrine but also from gut-derived peptides such as cholecystokinin and psychostimulant agents such as amphetamine, the appetite-suppressing and cognition-enhancing actions of which are associated with increased arousal and anxiety (Raybould, 2007). Thus, peripheral signals of satiety and arousal, which are conveyed to the brain through the autonomic nervous system and the NTS, may broadly diverge within the forebrain to activate distinct sets of neural substrates. In the case of OEA, such substrates appear to include the BLA, which may be involved in mediating the memory enhancing effects of OEA along with the paraventricular and supraoptic nuclei in the hypothalamus, which may be responsible for the anorexic actions of this compound (Rodriguez de Fonseca et al., 2001).

Campolongo and coworkers (2009) described how the post-training administration of OEA enhanced memory of training in a water-maze, a task that assesses memory for spatial context, as well as inhibitory avoidance, an aversively motivated task for which memory of context is an essential component, strongly suggests that OEA may have a general memory-modulatory influence that is not restricted to any specific kinds of experiences associated with its endogenous release. Prior studies of the effects of adrenergic receptor agonists indicate that such treatments enhance memory of a wide variety of types of training experiences (McGaugh and Roozendaal, 2002). Such findings, as well as the present findings, thus support the view that endogenously released modulators of memory consolidation act independently of the kinds of information associated with their activation.

Previous studies have shown that OEA is a high-affinity PPAR-α agonist (Fu et al., 2003) and that activation of this nuclear receptor accounts for most pharmacological actions of OEA—including prolongation of satiety (Astarita et al., 2006), stimulation of lipolysis (Guzman et al., 2004), and decrease in body weight gain (Fu et al., 2008). Campolongo (2009) also showed that PPAR-α is responsible for the memory-enhancing effects of OEA, because such effects are mimicked by two distinct PPAR-α agonists, are absent in mutant PPAR-α−/− mice, and are not affected by the TRPV-1 antagonist capsazepine. Moreover, although PPAR-α is expressed in the central nervous system (Moreno et al., 2004), the fact that OEA does not cross the blood–brain barrier indicates that the compound acts on PPAR-α located in peripheral tissues. PPAR-α is a key regulator of lipid metabolism and is thought to serve important functions in the absorption, storage, and use of

dietary fat (Evans et al., 2004; Bookout et a., 2006). Camopolongo's findings (2009) broaden the functional reach of this nuclear receptor to include a previously unsuspected role in the regulation of memory, and raise questions concerning the precise cellular localization of PPAR-α involved in the cognitition-enhancing effects to OEA and the target genes responsible for such effects.

Inhibitors of fatty acid amide hydrolase (FAAH) increase endogenous levels of anandamide (a cannabinoid CB1-receptor ligand) and oleoylethanolamide and palmitoylethanolamide (OEA and PEA, ligands for alpha-type peroxisome proliferator-activated nuclear receptors, PPAR-alpha) when and where they are naturally released in the brain. Using a passive-avoidance task in rats, Mazzola and coworkers described that memory acquisition is enhanced by the FAAH inhibitor URB597 or by the PPAR-alpha agonist WY14643, and these enhancements were blocked by the PPAR-alpha antagonist MK886. These findings demonstrate novel mechanisms for memory enhancement by activation of PPAR-alpha, either directly by administering a PPAR-alpha agonist or indirectly by administering a FAAH inhibitor.

The amygdala and the histaminergic control of different fear motivated memories

Evidence indicates that histamine modulates learning and memory in different types of behavioural tasks; however, the exact nature of this modulation and its mechanisms are controversial. Furthermore, emotions are able to influence memory processing in a crucial way through the involvement of the amygdala. Recently, Daher and Mattioli (2015) descrivbed how a pre-test intra-amygdala microinjection of histamine induced anxiolytic-like responses examined in the elevated-plus maze, without interfering in mnemonic processing. Concerning the IA task, step-through retention latencies increased in all groups compared with their respective trials, except in the animals microinjected with histamine before the retention test. Thus, histamine caused statistically significant amnesia during the session repeated 24 hours after training without drugs. These results contribute further evidence of the distinct histaminergic influence on different emotional pathways. But depending on timing and memory task, intra-BLA histamine differently modulates mnemonic processes. Benetti and colleagues (2015) show that the integrity of the brain histaminergic system is necessary for long-term memory LTM but not short-term memory of step-down inhibitory avoidance and that the acute histamine depletion in hippocampus or basolateral amygdala impairs long-term memory of that task (Cangioli et al., 2002; Passani et al., 2001). Importantly, differently from the previous study, histamine infusion into either structure restores in histamine-depleted rats and the restoring effect in the basolateral amygdala occurs

even when hippocampal activity was impaired. Central histamine seems also to control aversive memory extinction: Izuiqerdo and colleagues studied the action of the histamine enhancer SKF91488, the histamine H2 receptor agonist, dimaprit, and the histamine H2 receptor antagonist ranitidine microinfused after an extinction training session of contextual fear conditioning or after that of inhibitory avoidance into hippocampus, BLA or vmPFC on the consolidation of extinction. SKF91488 and the histamine H2-receptor agonist, dimaprit enhanced, and the H2 antagonist, ranitidine blocked memory consolidation of fear extinction in all three areas of the brain (Bonini et al., 2011 and Fiorenza et al., 2012). Another example of the histaminergic control of memory was fornished by Benetti and coworkers (2015): this work suggests that the memory deficit induced by early postnatal maternal deprivation in rats may at least in part be due to an impairment of histamine H3 receptor-mediated mediated mechanisms in the basolateral amygdala.

Aim III

Campolongo and coworkers (2009) described how the post-training administration of OEA enhanced memory of training in a water-maze, a task that assesses memory for spatial context, as well as inhibitory avoidance, an aversively motivated task for which memory of context is an essential component, strongly suggests that OEA may have a general memory-modulatory influence that is not restricted to any specific kinds of experiences associated with its endogenous release.

Evidence indicates that histamine modulates learning and memory in different types of behavioural tasks; however, the exact nature of this modulation and its mechanisms are controversial. Furthermore, emotions are able to influence memory processing in a crucial way through the involvement of the amygdala.

Provensi et al., (2014) pointed out the crucial role of central histaminergic stystem in modulating OEA anorexiant effect. Recently Benetti and co-workers (2015) described the crucial effect of the lack of central histamine in consolidation of IA paradigm: this study evidenced that central histamine presents a fundamental and differentiated role in memory depending on timing and brain region involved. The same authors evidenced that the histamine in the BLA is able to sustain IA consolidation even when this amine is absent in the hippocampus.

In this study we study the role of the histaminergic system in the modulation of the pro-mnesic effect OEA in the consolidation of contextual fear conditioning.

Materials and methods III

Animals

Male Wistar rats (3 mounths old, 300–330 g) purchased from Envigo (Bresso, Italy). Animals were housed in the animal facility of NEUROFARBA-Section of Pharmacology and Toxicology, Università di Firenze, housed in a temperature-controlled room (22 ± 1°C) with a 12:12-h light-dark cycle (light on 0700–1900 h), at a constant temperature and humidity with standard diet (4RF21; Mucedola s.r.l., Milan, Italy) and freely available water. All procedures were conducted in accordance with the Council Directive of the European Community (2010/63/EU) of the Italian Decreto Legislativo 26 (13/03/2014), and National Institutes of Health guidelines on animal care and approved by veterinarian supervision.

Surgery

At least 1 week after their arrival, rats were anaesthetized (75 mg/kg ketamine plus 10 mg/kg xylazine) and placed on a stereotaxic frame (Stellar; Stoeling). A stainless steel cannula (22 gauge) was implanted in the LV and fixed to the skull by using dental cement, according to the following coordinates: anterior, −0.9 mm; lateral, −1.5 mm; ventral, −2.6 mm (Paxinos and Watson, 1998), and used for a-FMHis administration. Rats were also implanted bilaterally with 22-gauge guide cannulae 1 mm above 1 mm above the BLA. The coordinates were anterior, −2.4 mm; lateral, ±5.1 mm; ventral, −7.5 mm for the BLA (Paxinos and Watson, 1998). Animals were allowed 7 days to recover from surgery before behavioural and biochemical procedures. Animals were handled once daily for three consecutive days, and all behavioural procedures was conducted between 8:00 and 11:00 AM.

Correct Cannula Placements

Correct cannulae placement were verified by infusing a 4% (wt/vol) methylene blue solution over 30 s into the CA1 and vmPfcx region (1 µL per side) or the BLA (0.5 µL per side) the second day after the last behavioural procedure. Animals were killed 30 min later by an overdose of the anaesthetics; brains were withdrawn and stored in formalin. The spread of the dye was taken as an estimate of that of the drug infusions in the same animals. Placements were considered correct when the spread was 1 mm3 or less (de Carvalho Myskiw et al., 2013; Fiorenza et al., 2012) from the intended infusion sites, which occurred in 98% of the animals. Only behavioral data from these animals were analyzed. As explained in Myskiw et al., (2008), despite the uncertainties given by the unknown rate of solubility of the drugs used relative to methylene blue, this procedure is an improvement over the mere determination of the cannula tip location.

Contextual Fear Conditioning

Contextual fear conditioning was induced in a Skinner box module (29 × 31 × 26 cm, Modular Operant Cage; Coulbourn Instruments Inc., USA), equipped with a grid floor connected to a shock-delivery apparatus (Modular Operant Cage/Grid Floor Shocker E13-08; Coulbourn Instruments) and placed in an acoustically insulated room at 20 ± 1 °C, as in previous experiments (Baldi *et al.* 2007). The number of the electric shocks and the inter-shock interval duration was predetermined by a stimulus programming device (Scatola di comando Arco 2340, Italy). Illumination inside the room was 60 lux. The rat was left undisturbed for 3 min and subsequently seven 1-s 1-mA electric footshocks were administered at 30-s intervals. The footshock intensity was chosen according to previous published data from our laboratory (Cangioli *et al.* 2002; Giovannini *et al.* 2003). This is a strong enough footshock to guarantee retention at 72 hours post-acquisition without inducing generalization (Baldi *et al.* 2004). The rat was removed 2 minutes after the end of the stimulation, therefore spending a total time of 8 min inside the conditioning apparatus.

Freezing measurement

Seventy-two hours after conditioning, rats were again placed inside the conditioning apparatus in the soundproof room and left undisturbed for 6 min. The rats' behaviour was recorded by means of a closed circuit television system by an experimenter unaware of the animal's treatment. Freezing (immobility) was defined as the complete absence of somatic motility, with the exception of respiratory movements (Sacchetti *et al.* 1999). Measurements were performed with a stopwatch by personnel who did not know to which experimental group each animal belonged. Total cumulated freezing time (i.e. total seconds spent freezing during each 3 min period) was calculated and results expressed as percentage of freezing time. All behavioural tests were performed between 10:00 and 12:00 hours to avoid interference with the circadian rhythm (Kamin, 1957).

Drugs

The drugs used were α-FMHis (5 μg/uL) was synthesized at Abbott Laboratories, pyrilamine (0.9 μM) purchased from Sigma–Aldrich (UK), zolantadine (0.1 μM) purchased Tocris Bioscience (UK), OEA (Tocris Bioscience, Bristol, UK) was dissolved in saline/polyethylene glycol/Tween80 (90/5/5, v/v).

Infusion Procedure and experimental groups

At the time of drug microinfusions, the animals were gently restrained by hand, and the injection needle (30 gauge) was fitted tightly into the guides, extending 1 mm from the tip of the guide

cannulae. The injection needle was connected to a 10-µL Hamilton microsyringe, and the infusions were performed at a rate of 0.5 µL/30 s. The infusion cannula was left in place for an additional 60 seconds to minimize backflow. It was then carefully withdrawn and placed on the other side, where the procedure was repeated. The entire bilateral infusion procedure took approximately 90 seconds. In the first set of experiment: OEA (10 mg/kg) was infused immediately after fear conditioning, while controls received equivalent volumes of vehicle. In the second set of experiments α-FMHis (5 µg/µl) i.c.v. 24 hours before the contextual fear conditioning, controls received equal volumes of steril saline; OEA (10 mg/kg) was infused immediately after fear conditioning, while controls received equivalent volumes of vehicle. In the third set of experiments zolatadine (0.9 µM) and pyrilamine (0.9 µM) were infused intra-BLA bilaterally immediatelly after contextual fear conditioning; OEA (10 mg/kg) was infused 10 minutes after fear conditioning, while controls received equivalent volumes of vehicle

RESULTS III

Effect of the lipid-derived oleoylethanolamide (OEA) on consolidation of contextual fear conditioning and involvement of central histaminergic system: preliminary data

Campolongo and colleagues (2009) described OEA's facilitation in memory consolidation of different memory paradigms, such as inhibitory avoidance and Morris water maze when this substance is administrated pre-training, and pointed out how this effect is, at least in part, mediated by the activation of NTS that stimulates β-adrenergic receptors in BLA.

Firstly, we evaluated the effect of OEA (10 mg/kg i.p.) administered immediately after contextual fear condition (CFC) in satiated rats. Controls received an equivalent volume of vehicle. One-way ANOVA revealed a statistical difference across experimental groups ($F_{5\text{-}65} = 6.21$; $P<0.0001$).

Newman-Keuls MCT shows a significant difference in matter of time spent freezing between control group and rats given OEA i.p. immediately after conditioning: freezing times of controls reveal that the animals consolidated the aversive memory context-related of the fearful stimulus received during conditioning but OEA-treated rats present higher freezing times, meaning that the administration i.p. of the lipid-derived OEA is able to enhance aversive memory consolidation of contextual fear conditioning, confirming Campolongo and colleagues previous results (Campolongo et al., 2009; $P<0.05$).

To evaluate the role of the central histaminergic system in the cognitive effect of OEA, we infused i.c.v. α-FMHis 24 hours prior to fear conditioning to deplete releasable central histamine. Controls received the same volume of sterile saline. OEA (10 mg/kg) was administred i.p. immediately after conditioning, while control group received an equivalent volume of vehicle. One-way ANOVA revealed a statistical difference across experimental groups ($F_{5\text{-}65} = 6.21$; $P<0.0001$). The rats that received α-FMHis i.c.v. and vehicle i.p. showed a great variability that did not reach statistically significant, freezing time difference with respect to controls. Interestingly, the time spent freezing by the rats that received α-FMHis and OEA was not different from the α-FMHis controls and significantly shorter than rats given saline i.c.v. and OEA ($P<0.01$).

Hence, the treatment with α-FMHis 24 hours prior to test prevented the pro-mnesic effect of OEA, indicating that central histaminergic system is necessary for the effects of OEA on memory consolidation.

Given the requirement of the integrity of histaminergic neurotransmission in the BLA for OEA to exert its cognitive effects, we then investigated which histaminegic receptor subtypes are

involved. We therefore injected intra-BLA bilaterally a) the H1 receptor antagonist pyrilamine (0,9 μM) immediately after conditioning and OEA i.p. 10 minutes after training; b) the H2 receptor zolantadine (0.1 μM) immediately after conditioning and OEA i.p. 10 minutes after training. III) saline bilaterally intra-BLA immediately after conditioning and OEA i.p. 10 minutes after training; IV) saline bilaterally intra-BLA immediately after conditioning and vehicle i.p. 10 minutes after training. One-way ANOVA further described a significant difference in time spent freezing of PYR/OEA- and ZOL/OEA-treated rats compared to SAL/OEA given rats ($F_{5\text{-}65} = 6.21$; $P<0.0001$). A Bonferroni's MCT then showed that animals which received intra-BLA pyrilamine or zolantadine and OEA presented lower times spent freezing compared to animals that received saline intra-BLA and OEA ($P<0.001$), pointing out the importance of both histaminergic receptor types in the facilitation of CFC consolidation cause by i.p. administration of OEA.

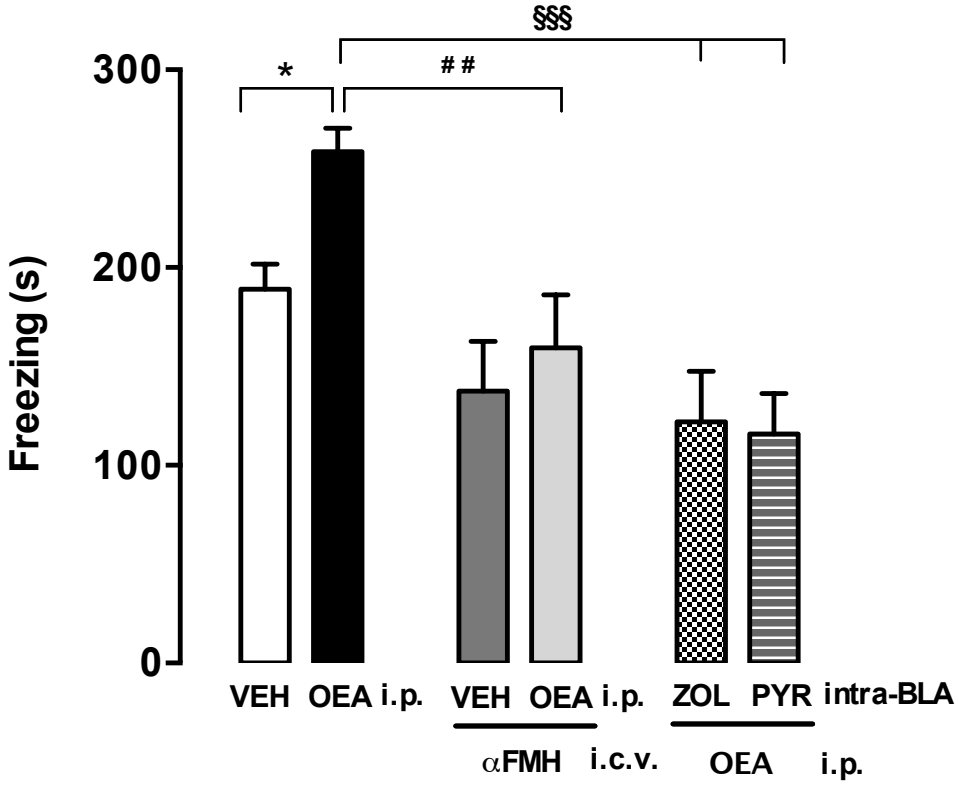

Figure III 1. Effect of oleoylethanolamide on consolidation of contextual fear conditioning and involvement of central histaminergic system.

Left part of the graph: freezing time (s) of rats given OEA (10 mg/kg, i.p.) immediately after conditioning. Retention test was carried out 72 hrs after condition. Time spent freezing of OEA-treated rats were significantly higher compared to controls that received equal volumes of vehicle. Data are expressed as means ± SEM of 8-12 animals for each experimental group; * P<0.05 vs respective controls; One-way ANOVA followed by Newman-Keuls MCT.

Central part of the graph: Rats were implanted with stainless infusion cannulas and received α-FMH i.v.c 24 hours prior to contextual fear conditioning. Rats received OEA (10 mg/kg, i.p.) immediately after conditioning. Retention test was carried out 72 hrs after conditioning. Time spent freezing of α-FMH-treated rats and that received OEA are significantly lower compared to OEA-treated rats. Data are expressed as means ± SEM of 8-12 animals for each experimental group; ## P<0.01 vs. OEA-treated rats; One-way ANOVA followed by Newman-Keuls MCT.

Right part of the graph: Rats were implanted with stainless infusion cannulae and received zolantadine or pyrilamine immediately after contextual fear conditioning; controls received equal volumes of sterile saline. 10 minutes after contextual fear conditioning rats received OEA i.p. or vehicle. Retention test was carried out 72 hrs after conditioning. Time spent freezing of ZOL- and PYR-treated rats and that received OEA are significantly lower compared to OEA-treated rats. Data are expressed as means ± SEM of 8-12 animals for each experimental group; §§§ P<0.001 vs. OEA-treated rats; One-way ANOVA followed by Newman-Keuls MCT.

CONCLUSION III

Endocannabinoids (ECs) are neuromodulators and immunomodulators. Among them, it exists a class called EC-like ligands (ECLs), which includes oleoylethanolamide (OEA). These molecules act as anti-inflammatory mediators (Du *et al*., 2011; Sun *et al*., 2007) and antioxidants (Marsicano *et al.,* 2002). They are also involved in neuro-protection (Scuderi *et al.,* 2012; Viscomi *et al.,* 2009), and act as retrograde signals modulating LTP (long-term potentiation) and LTD (long-term depression), two alternative forms of synaptic plasticity underlying learning and memory (Iremonger *et al*., 2011). OEA is an endogenous ligand for peroxisome proliferator-activated receptor-α (PPAR-α) in the gut, recruiting local afferents to the vagus nerve (Campolongo *et al.,* 2009). Compolongo and colleagues (2009) described that OEA, along with the control on feeding behaviour, has also a fundamental role in modulating memory consolidation via a PPAR- α-mediated mechanism that activates NTS and BLA. Our present results evaluated effect of the post-training i.p. administration of OEA in satiated rats on consolidation of contextual fear conditioning paradigm: OEA-treated rats demonstrated a stronger memory for the aversive stimulus received during the contextual fear conditioning, as showed from the longer time spent freezing during test session compared to controls. These data confirm Campolongo's evidences (2009) and corroborate the hypothesis that OEA is critically involved in memory consolidation, fundamental in nature for the ability to remember context associated to aversive stimuli.

Among the central signals required to fully exert OEA modulation of feeding, Provensi and co-workers (2014) described that central histaminergic system has a crucial role, as mice lacking histamine, due to both acute or chronic depletion, showed a decreased hypophagic effect compared to their littermates. We here further demonstrate that OEA requires central histamine also to exert its facilitatory action on memory: as a matter of fact, in rats acutely deprived of central histamine the pro-mnemonic effect of OEA on CFC consolidation is blunt compared to OEA-treated animals with an intact histaminergic system.

Basolateral amygdala has been largely studied as fundamental in modulation of different types of memory and in particular it has been linked to memories with strong emotional values (Roozendaal and McGaugh, 2011). It is also known a large variety of agents modulate memory by acting on the basolateral amygdala, such as adrenaline (Williams *et al.,* 1998; Campolongo *et al.,* 2009), corticosterone (McReynolds *et al.,* 2010) and naloxone (Quirarte *et al.,* 1998). The basolateral amygdala also received histaminergic projections (Haas *et al.,* 2008) and histamine strongly modulates excitatory synaptic transmission in this area (Jiang *et al.,* 2005). Many studies show the involvement of histamine in modulation of consolidation of different types of memory

(Benetti *et al.,* 2015a; 2015b; Baldi *et al.,* 2005), mostly activating H2 receptors but also via inhibiting the H3 auto-receptor (Cangioli *et al.,* 2008). In agreement with Campolongo's report (2009), we hypothesized that histamine in the BLA could, at least in part, modulate the effect of OEA in contextual fear conditioning consolidation. The infusion of H1-antagonist, pyrilamine, and H2-antagonist, zolantadine, infused immediately after the conditioning were indeed able to blunt the promnesic effect of OEA on aversive memory consolidation.

The present results demonstrate that central histaminergic histamine is necessary for the lipid-derived OEA to fully exert its pro-cognitive effect on memory consolidation: central histamine has been largely involved in modulation of different types of memory, aversive ones (de Almeida and Izquierdo, 1986; Benetti *et al.,* 2015a; Baldi *et al.,* 2005; Cangioli *et al.,* 2008) but also memories not derived from fearful stimuli such as novel object or social recognition (Giannoni *et al*, 2009; Giovannini *et al*, 1999). Because Provensi and co-workers (2014) proved that this amine is crucially involved in the modulation of the hypophagic effect of OEA, we can propose that central histamine is also fundamental in mnemonic processes linked, in nature, to animlas' ability to remember food sources or avoid dangers foraging-related. This work furnished new insights about the complex interplay of peripheral and central stimuli that mediate the effect of OEA in mnemonic processes and proposes central histamine as a new pharmacological target to amplify OEA effects, not only in feeding (Provensi *et al.,* 2014), but also in memory.

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