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Brain histamine as a gateway for the improvement of stress-induced maladaptive behaviours and social memory

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Abstract

Stress-related disorders are common and debilitating conditions characterised in part by affective manifestations associated with cognitive and behavioural alterations. These disorders could be chronic and severe at some point in life (Tolentino and Schmidt 2018).

Stress is a triggering factor for both anxiety and depression, therefore stress modulation may represent an winning strategy in the treatment of stress-associated disorders (Moritz et al. 2020). In addition, understanding the neurophysiological aspects implicated in the etiology of stress-associated disorders may ultimately contribute to their treatment.

Lately, nutritional interventions to ameliorate stress-induced cognitive deficits is gaining worldwide interest. Since the discovery of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) in 1929 by George and Mildred Burr (Burr 1929), research on ω-3 PUFAs became an appealing topic ranging from their role in cardiovascular risk and more recently neuropsychiatric pathologies such as depression and anxiety, cognitive decline or neurodegenerative diseases (Bazinet and Layé 2014, Joffre et al. 2014, Coulombe et al. 2018). The relevance of lipids in brain function is illustrated by the fact that the central nervous system (CNS) has the highest concentration of lipids in the organism after adipose tissue. Among these lipids, the brain is particularly enriched with PUFAs represented by the ω-6 and ω-3 series.

In animal models, it has been shown that transient or maternal ω-3 PUFA-deficient diet induces depressive and anxiety-like symptoms (Carrié et al. 2000, DeMar et al. 2006, Bondi et al. 2014) as well as abnormal social behaviour (Lafourcade et al. 2011) in adolescent and adult offspring. However, the mechanisms underlying the effects of ω-3 PUFA-deficient diet on emotional behaviour remain largely unknown.

In this thesis, I evaluated the role of a diet enriched with ω-3 PUFA (EPA and DHA) and vitamin A, another nutrient that plays a key role in the regulation of synaptic plasticity and in learning and memory in adult rats (Lane and Bailey 2005), by using a preclinical model of adolescent stress, the social instability paradigm. We studied the effects of the enriched diet in the cognitive and neurochemical deficits induced by this kind of stress. The results showed that the supplemented diet had beneficial effects preventing cognitive and neurochemical alterations of juvenile rats tested immediately after the stressful procedure. but also when rats were tested as adults. Furthermore,

the detrimental behavioural and neurochemical effects of adolescent stress, as well as the protective effect of the enriched diet, were maintained throughout adulthood, long after the exposure to the stressful environment was terminated.

Histamine is implicated in arousal, awakening and maintenance of wakefulness and has a pivotal role in the maintenance of high vigilance that is required for cognitive processes (Thakkar 2011, Thakkar et al. 1999). Not surprisingly, current research is providing evidence that malfunctioning of the histaminergic system is associated with neuropathological disorders (Shan, Bao and Swaab 2017). For this reason, in the second part of the thesis I evaluated the role of the brain histaminergic in the protective effects of the ω-3 PUFA and Vitamin A enriched diet. To this end I used genetically modified mice that do not synthesise histidine decarboxylase ($HDC^{-/-}$, the only enzyme responsible for histamine synthesis) and $HDC^{+/+}$ mice. These were exposed to chronic social defeat stress, a well-characterised preclinical model of anxiety and depression (Krishnan and Nestler 2011). The results demonstrated that the enriched diet prevented the memory impairment and social avoidance behaviour induced by stress only in animals whose histaminergic system is intact, thus supporting our hypothesis on the key role of the histaminergic system in the beneficial effects of this diet.

Oleoylethanolamide (OEA) is derived from a monounsaturated fatty acid oleic acid (OA), which has beneficial effects on intestinal metabolism (Lama et al., 2020) regional fat distribution (Sarro-Ramírez et al. 2013), inflammation (Piomelli 2013). Furthermore, OEA has precognitive effects (Campolongo et al., 2009; Provensi et al., 2017)

Previous preclinical studies in our laboratory showed that the central histaminergic system is essential for the central effects of OEA such as the hypophagia (Provensi et al. 2014), improved memory for aversive events (Provensi et al. 2017) and antidepressant-like action (Costa et al. 2018). For this reason, I also evaluated the effect of a chronic administration of OEA in mice subjected to the chronic social defeat stress. The results obtained showed that treatment with OEA prevents behavioural impairments induced by chronic stress in $HDC^{+/+}$, but not in $HDC^{-/-}$ mice, further supporting the role of the histaminergic system in the beneficial effects of OEA.

In the last part of my thesis I investigated the role of central histaminergic system on social recognition memory. Social memory is one of the crucial components of episodic memories. Among the four identified histamine receptors, H_3 receptors are

predominantly expressed in the central nervous system, act as autoreceptors as well as heteroreceptors, and control presynaptic release of histamine and other neurotransmitters (Haas, Sergeeva and Selbach 2008).
The results demonstrated that acute and pharmacologically or genetically histamine depleted mice had long term, but not short-term memory impairment. However, histamine deprived mice treated with a selective H_3 receptor agonist VUF16839, that supposedly decreases histamine and other neurotransmitters' release showed short-term memory impairment as well. We interpret this finding as indicative of the involvement of H_3 heteroceptors on non-histaminergic cells being involved in the amnesic effect. Indeed, treatment with the acetylcholinesterase inhibitor, donepezil, prevented the amnesic effect of VUF16839.
The results presented in this thesis strongly suggest that central histaminergic system plays a crucial and fundamental role in mediating the beneficial effects of nutritional compounds such as the ω-3 PUFA and vitamin A enriched diet or OEA on behavioural impairments induced by stress.
I also contributed to the understanding of the role of the central histaminergic and the interplay with other neurotransmitter systems on social recognition memory.

Social behaviour: From social stress to social memory

We live in a world that is largely socially constructed and we are constantly involved in, and fundamentally influenced by a large array of complex social interactions.

In a broad sense, social behaviours can be defined as any modality of communication and/or interaction between two conspecifics of a given species and are observed in species as simple as single-celled microorganisms to species as complex as humans (Crespi 2001, Ebstein et al. 2010). Social behaviours, no matter whether cooperative or competitive, have been selected for and have persisted throughout evolutionary history due to their contributions toward increasing survival and reproductive fitness. Social behaviours displayed at the inappropriate time or place or of inappropriate intensity can have detrimental effects on both the individuals and a social group as a whole. Mating, or sexual reproduction, is a clear example of an absolutely required social behaviour for reproductive fitness, as it is the substrate for genetic heritability across generations. Aggression is an example of a competitive social behaviour where the winner of an aggressive encounter is provided greater access to resources, including territories or mating opportunities, resulting in a greater chance of survival and reproductive success. Social group living, termed sociality, also increases reproductive fitness due to group association offering greater capabilities for threat defence, resource acquisition, and opportunities for mating (Silk 2007).

Social behaviours are essential for the health, survival, and reproduction of animals; conversely, impairment in social function is a prominent feature of several neuropsychiatric disorders, such as autism spectrum disorders and schizophrenia (Chen and Hong 2018).

Traditionally, the emphasis has been placed on basic neuronal networks underlying a specific type of behaviour, as well as hormonal or pharmacological manipulations of this behaviour. More recently, reciprocal links with other behavioural systems have received increasing attention. Examples of these links are the effects of stress (acute, chronic, or early in life), trait or state anxiety, cognitive skills, and impulse control on positive and negative social interactions, and vice versa the effects of positive and negative social experiences on stress system (re-)activity, mood, and cognition. The field of translational social neuroscience comes with certain challenges. Foremost, neuroscientists will benefit from knowing the behavioural ecology of their model animal or its ancestors far more intimately than is typically necessary for the study of depression and anxiety or learning and memory (Lukas and de Jong 2015).

Stress: social behaviour and resilience

Definition and neurobiology of stress

Stress has a different meaning for different people under different conditions. The first and most generic definition of stress is that proposed by Hans Selye: "Stress is the non-specific response of the body to any demand" (Selye 1936).

The *Diagnostic and Statistical Manual of Mental Disorders* (DSM-V) recognizes two stress disorders: acute stress disorder and posttraumatic stress disorder (PTSD). For the diagnosis of acute stress disorder, the individual, while experiencing the trauma or after the event, must have at least three of several dissociative symptoms, such as a subjective sense of numbing, detachment, or absence of emotional responsiveness; reduction in awareness of surroundings; depersonalization; or dissociative amnesia. Following the trauma, the traumatic event is persistently re-experienced, the individual avoids stimuli that may arouse recollections of the traumatic event, and they have anxiety or increased arousal. PTSD is defined as a condition in which a traumatic event is persistently re-experienced in the form of intrusive recollections, dreams, or dissociative flashback episodes. Cues to the event lead to distress and are avoided, and there are symptoms of increased arousal; the full symptom picture must be present for more than one month, and the disturbance must cause clinically significant distress or impairment in social, occupational, or other areas of functioning (Fink 2009).

Stress is usefully viewed from a biological perspective; accordingly, it involves activation of neurobiological systems that preserve viability through change or allostasis. Although they are necessary for survival, frequent neurobiological stress responses increase the risk of physical and mental health problems, perhaps particularly when experienced during periods of rapid brain development (Gunnar and Quevedo 2006).

Physiological and neurochemical approaches have elucidated the way in which stress is controlled by two major neuroendocrine systems, the hypothalamic-pituitary- adrenal (HPA) axis (Stratakis and Chrousos 1995) and the sympathetic-adrenomedullary (SAM) limb of the autonomic nervous system (ANS) (Frankenhaeuser et al. 1986).

The SAM system is a component of the sympathetic division of the autonomic nervous system, releasing epinephrine (adrenaline) from the medulla or centre of the adrenal gland. Increases in circulating epinephrine facilitate rapid mobilization of metabolic resources and orchestration of the *fight/flight* response (Cannon 1929). The HPA system, in contrast, produces glucocorticoids (cortisol in humans, corticosterone in rodents; hereafter GCs) which are steroid hormones. Unlike epinephrine, which does not cross the blood-brain barrier to a significant degree, the

brain is a major target of GCs (Bohus, De Kloet and Veldhuis 1982). Also, unlike epinephrine, GCs production takes some time (approximately 25 minutes to peak levels), and many of its impacts on the body and brain occur through changes in gene expression (de Kloet, Rots and Cools 1996). Consequently, the effects of GCs are slower to develop and continue for longer periods (de Kloet et al. 1996). The role of the HPA system in stress is complex, and its functions are not fully captured by reference to the fight/flight response (Sapolsky, Romero and Munck 2000). Regulation of both the SAM and HPA systems converges at the level of the hypothalamus, which integrates autonomic and endocrine functions with behaviour (Palkovits 1987). Furthermore, inputs to the hypothalamic nuclei that orchestrate HPA and SAM responses to psychosocial stressors involve cortico-limbic pathways (Gray and Bingaman 1996).

Social stress

Social stress, a common stressor readily translated across species, is a recurrent factor in the life of all social species (Von Holst 1998). The effects of stress exposure and consequent trajectory depend on the nature of the stressor, the severity, duration (acute vs. chronic), sex/gender, genetics, timing of exposure (early life, adolescence, adulthood or aging) as well as the perception of the stressor by the individual, for example, stressor controllability dramatically affects resilience versus vulnerability as an outcome (Maier and Watkins 2005, Amat et al. 2010, Lucas et al. 2014).

The nature of the stressor is important; physical (e.g., electric, chemical and terminal stimuli, as well as surgeries), psychological (e.g., restraint, exposure to novel environments, forced swimming) or social (when the stimulus is represented by the behaviour of a conspecific or of its products) stressors elicit different responses (Bartolomucci 2007). The duration of the exposure to a stressor is important. A stressor may be defined as acute (e.g., minutes, hours or a single event), intermittent (e.g., repetition over time of an acute stressor) or chronic (continuous exposure to a stressor) (Kusnecov and Rossi-George 2002). Acute stressor will, by definition, immediately activate the stress–response. The termination of the activated response is not always complete and intermittent or repeated perturbations may last weeks or even years in specific situations (Koolhaas et al. 1997, Yehuda 2002). In general, an intermittent or chronic exposure is needed to translate a stress–response into a pathological condition (van Kampen et al. 2002).

In rodents, acute stress typically leads to reduced social behaviours and increased aggression, including antisocial behaviours such as bite counts that exceed species-typical levels (de

Almeida and Miczek 2002). This fits with the concept of acute stress as a 'flight or fight' response and implies that brief acute stressors mobilize resources to cope with the situation (Sandi and Haller 2015).

Chronic stressors are defined as persistent events which require an individual to make adaptations over an extended period of time. When stress becomes chronic, one experiences emotional, behavioural, and physiological changes that can put one under greater risk for developing a mental disorder and physical illness (Krieger and Loch-Caruso 2001). Chronic stress reduces social motivation and social interactions in a variety of sociability tests (van der Kooij et al. 2014, Wood et al. 2003). However, although chronic stressors generally reduce sociability, social isolation stress actually enhances social interest (van den Berg et al. 1999), probably because long-term deprivation from social contacts increases interest in social partners.

Chronic social stressors that involve fighting that leads to defeat and subordination have been shown to downregulate aggressiveness in various species. Conversely, repeated victories which are accompanied by reduced physiological stress responses, but which can be considered stressful because they involve recurrent exposure to social conflicts may result in exacerbated and abnormal aggression (Nephew and Bridges 2011, Miczek, de Boer and Haller 2013).

Chronic stress paradigms in rodents

Stress models that cover a range of neurodevelopmental periods have been applied to investigate the long-term impact of stress on adult social behaviours. Social motivation (sociability) was disrupted in adulthood by prenatal (de Souza et al. 2013), neonatal (Franklin et al. 2011) and juvenile (Naert (Naert et al. 2011, Vidal, Buwalda and Koolhaas 2011) exposure to stressors. Prenatal stress (DE Souza, 2013), neonatal stressors-maternal separation (Wang, Shao and Wang 2015a), and early deprivation (Jia et al. 2009) and peripubertal exposure to physical stressors (Márquez et al. 2013) inhibited social interactions in adulthood. Juvenile social stressors (post-weaning social isolation (Workman et al. 2011) and early subjugation (Wommack et al. 2004) either did not affect this aspect of social behaviour or, in one study (Shimozuru et al. 2008), increased adult social interactions. Early life stressors decrease measures of social motivation, reduce the expression of social behaviours, increase aggressiveness and promote the development of antisocial features, but the specific consequences depend on the timing and type of the early stressor. Although these changes can be problematic for human individuals and societies, from an evolutionary perspective they may

be interpreted as mechanisms through which early adversity prepares the organism to endure similar adversities later in life (Gluckman, Hanson and Beedle 2007).

The social environment interacts with stress on almost every front: social interactions can be potent stressors; they can buffer the response to an external stressor; and social behaviour often changes in response to stressful life experience. Widely used models of social stress in rodents include social subordination, crowding, isolation, and social instability (Figure 1) (Beery and Kaufer 2015)

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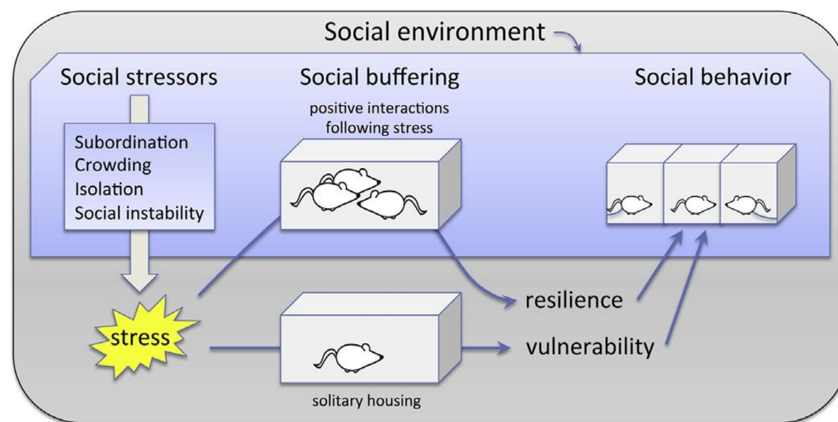


Figure 1. Schematic representation of the levels at which the social environment impacts (Beery and Kaufer 2015).

Chronic social defeat stress.

The most common stressors in man are of a psychological or social nature (Kessler 1997, Kessler, Price and Wortman 1985, Björkqvist 2001), and therefore, using social conflict between members of the same species to generate stress has an obvious advantage over animal models that require aversive physical stimuli such as electric foot shock, restraint, water or food deprivation, or cold exposure. Several studies have shown that subordination stress (social defeat) is an important factor that may lead to psychopathological changes (Björkqvist 2001, Fuchs and Flügge 2002).

In rodents, the most prominent model of stressful social interaction is social defeat. Social defeat is typically induced by a version of the resident-intruder test in which a test subject is paired with a dominant resident in its home cage. Dominance may be assured by size, prior history of winning, strain of the resident, and/or prior housing differences (Martinez, Calvo-Torrent and Pico-Alfonso 1998). These animal models are based on a physical contact phase, where an intruder animal is physically exposed to an attacking resident aggressor, and/or a sensory contact phase during which the intruder is kept in visual, auditory and olfactory contact

with the dominant con-specific in order to maintain psychological stress for the intruder animal (Hammels et al. 2015). The total duration of exposure to the aggressor in social defeat paradigms may vary from minutes to weeks, broadly classified as acute and chronic exposure (Martinez et al. 1998). Acute social defeat is defined as a single social confrontation for a few minutes (Berton et al. 1999), or several short interactions on the same day (Huhman et al. 2003), whereas social conflicts in chronic paradigms typically last from 10 days (Berton (Berton et al. 2006) up to 40 days (Chang et al. 2009, Bartolomucci et al. 2001). The sensory contact phase has been implied to maintain psychological stress for the intruder animals for a prolonged period. Intruder animals can be exposed to a sensory contact period either before (Tornatzky and Miczek 1993) or after the physical contact period (Berton et al. 2006). The duration of the sensory contact phase can vary from 10 min (Tornatzky and Miczek 1993) to 24 h (Berton et al. 2006).

In the short-term, social defeat produces changes in heart rate, hormone secretion, and body temperature, with longer-term impacts on a wide variety of additional outcomes including activity, social behaviour, drug preference, disease susceptibility and others (Martinez et al. 1998, Sgoifo et al. 1999, Peters et al. 2012). Repeated episodes of social defeat, particularly if they are unpredictable and uncontrollable, amplify and prolong these behavioural and neurobiological consequences (Tornatzky and Miczek 1993, Yap et al. 2006). Repeated defeat followed by individual housing results in long term impaired social memory, decreased social interaction and diminished anticipation for a sucrose reward up to 3 months after the last defeat experience,

Unlike physical stressors such as restraint, social defeat does not appear to be susceptible to habituation or sensitization (Tornatzky and Miczek 1993, Sgoifo et al. 2002). Social defeat stress has profound effects on hippocampal morphology and function (McEwen and Magarinos 2001, Buwalda et al. 2005, Mirescu and Gould 2006, McEwen 2012). These effects include reduction in hippocampal volume (Czéh et al. 2001) related to dendritic remodelling and reduced neurogenesis (Magariños et al. 1996, Gould et al. 1998)et al., 1996; Gould et al., 1998), as well as attenuated induction of long-term potentiation and suppressed facilitation of long-term depression in the CA1 region of the hippocampus (Von Frijtag et al. 2001). Social defeat also alters the ratio of mineralocorticoid to glucocorticoid receptors in the hippocampus (Buwalda et al. 2001, Veenema et al. 2003). As with most of neurobiological research, attention has centred on neurons as the mediators of the biological embedding of the social world. However, following recent reports on the effects of stress (in general, and particularly social stress) on astrocytes, oligodendrocytes and microglial cells, it has become clear that glial cells

are likely to play a role in this process, and deserve more attention in future studies (Braun et al. 2009, Wohleb et al. 2011, Araya-Callís et al. 2012, Chetty et al. 2014)

Crowding and isolation

Housing density affects rodent behaviour, and both crowded and isolated social environments have been used as stressors in rodents. Crowding is a naturalistic stressor especially for social or gregarious species that relates to high population density and resource competition in the field. In house mice, several studies have shown that crowding can impair reproductive function and may be part of population size regulation (Christian and Lemunyan 1958, Christian 1971). In the laboratory, crowding typically consists of large numbers of mice or rats (e.g. >6 rats/cage (Brown and Grunberg 1995, Reiss et al. 2007) with ad libitum access to resources such as food and water. Crowding must be somewhat extreme to induce stressful outcomes, as group housing (e.g. 4-6 rats or 12 mice in a sufficiently large area) is often used as a key component of environmental enrichment (Sztainberg and Chen 2010, Simpson and Kelly 2011). Social crowding has been shown to impact many different physiological outcomes in male mice, rats, and prairie voles. These include changes in organ weights, hormone secretion, HPA reactivity, pain sensitivity, telomere length, and cardiac outcomes (Gamallo et al. 1986, Gadek-Michalska and Bugajski 2003, Kotrschal, Ilmonen and Penn 2007, Grippo et al. 2010, Tramullas, Dinan and Cryan 2012, Puzserova et al. 2013).

At the opposite extreme, solitary housing can be a potent stressor for social species. Social isolation is employed as a stressor in previously group-housed mice and rats (Heinrichs and Koob 2006); in both species, extended (2-13 week) solitary housing produces an “isolation syndrome” particularly in females, consisting of hyperadrenocorticism, reduced body weight, altered blood composition, and enhanced pain responsiveness among other outcomes (Hatch et al. 1965, Valzelli 1973). These changes coincide with alterations in behaviour including aggression, mating behaviour, learning, and pain sensitivity (Valzelli 1973). More recent studies have added a host of additional physiological outcomes related to stress and depressive behaviour, including changes in dopamine signalling in different brain regions (Heidbreder et al. 2000), altered heart rate and cardiac function (Carnevali et al. 2012), and neurogenesis (Stranahan, Khalil and Gould 2006, Lieberwirth and Wang 2012). Which outcomes are affected by isolation depend in part on the age at which isolation occurs (Hall 1998), and there are sex differences in the effects of social isolation. These suggest that isolation may be stressful for females but not necessarily to the same extent for males (Hatch et al. 1965, Palanza 2001, Palanza, Gioiosa and Parmigiani 2001). Assessing the impacts of both isolation and crowding

share the problem of what to consider as the control comparison, as anxiety and other behavioural outcomes vary along a continuum of group sizes (Botelho, Estanislau and Morato 2007).

Chronic restraint stress

Chronic restraint stress (CRS) has been used widely to study the morphological, hormonal, and behavioural alteration in several brain regions in rodents, such as the hippocampus, prefrontal cortex, amygdala, and nucleus accumbens because it is inexpensive and relatively easy to implement (Buynitsky and Mostofsky 2009). Restraint stress is generally induced by keeping the animals in a cylindrical or semi-cylindrical tube with ventilation holes for 120-180 min. (Padovan and Guimarães 2000, Campos et al. 2010). The procedure can be used to induce either acute or chronic stress (7-21 days). A disadvantage of the CRS model is the habituation of rats or mice to repeated exposure to homotypic restraint stressors; the response of plasma corticosterone, the major glucocorticoids in rodents, to the final stressor is diminished in animals that had been stressed for 14 days (Ma and Lightman 1998, Martí and Armario 1998, Herman 2013). The duration of CRS may differentially affect learning/memory and hippocampal CA3 dendritic atrophy (Luine et al. 1996). Depending on duration and intensity of chronic stress, some studies report that exposure of animals to CRS induces depression-like behaviours such as anhedonia (decreased sucrose preference) (Aboul-Fotouh 2013, Chiba et al. 2012, Bravo et al. 2009), which is a core symptom of human depression (Hill et al. 2012, Willner 2005).

Social instability

Some studies employ both crowding and isolation in alternation (for example, 24 h of each for 2weeks), as a model for chronic social instability (Haller et al. 1999, Herzog et al. 2009). Social instability has particularly been used as a social stressor for female rats, for whom crowding alone and social defeat are not always effective stressors (Palanza 2001). In the crowding phase, different social groups consisting of different numbers of males and females are formed. Females exposed to this variable social environment show increased adrenal weight, increased corticosterone secretion, decreased thymus weight, and reduced weight gain relative to females housed in stable male-female pairs (Haller et al. 1999). A second study replicated these findings and demonstrated that social instability also induced dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis (elevated luteinizing hormone, prolactin, and disrupted oestrus cycles), and reduced sucrose preference and food intake (Herzog et al. 2009). This stressed

phenotype persisted for several weeks without habituation and led to a depressive-like phenotype. Prior history of social instability in the form of early-life separation from the mother also exacerbates vulnerability to later life chronic subordination stress (Veenema et al. 2008).

Social instability stress in adolescence

Adolescence represents a time of transition to independence during which significant lifestyle changes occur (Casey et al. 2002, Arnett 2000), and it is believed to be a critical period for the programming of future adult behaviours (Sawyer et al. 2012). Although there are no definite markers for the adolescent period, in mice and rats adolescence is generally considered to be from post-natal day (PND) 21–60, and in humans from ages 12 to 18 (Spear 2000b). Significant changes in neuroendocrine, neurodevelopmental and behavioural systems occur during adolescence.(Spear 2000b, Spear 2000a, Romeo 2010, McCormick and Zovkic 2009, Green and McCormick 2013). For instance, there are changes in reward circuitry that render both rodent and human adolescents less sensitive to the aversive effects of drugs of abuse (Doremus-Fitzwater, Varlinskaya and Spear 2010). Behaviourally, both rodents (Spear 2000a, Adriani and Laviola 2003, Douglas, Varlinskaya and Spear 2004) and humans (Spear 2000a, Forbes and Dahl 2010, Romer 2010, Steinberg 2008) show increased risk-taking (Steinberg 2008), social activity (Forbes and Dahl 2010, Vanderschuren, Achterberg and Trezza 2016) and impulsivity (Adriani and Laviola 2003, Romer 2010) across the adolescent period. Cognitive changes have also been demonstrated at this time of life (Yurgelun-Todd 2007) especially in relation to executive function (Blakemore and Choudhury 2006) and cognitive control (Kuhn 2006).

Social interactions are highly rewarding in adolescence (Douglas et al. 2004), and time spent in social play is highest during adolescence (Sachser, Dürschlag and Hirzel 1998, Meaney and Stewart 1981). Consistent with the latter, the effects of social isolation are greatest when applied during adolescence (Panksepp and Beatty 1980, Einon and Morgan 1977), and the social deprivation involved with social isolation has been an effective model to investigate psychopathology. McCormick et al. chose to develop a milder model of adolescent stress using social instability, on the basis that it might allow for broader relevance for some aspects of adolescent development in people. They used the combination of repeated daily 1 h isolation (confinement to a small, ventilated container) followed by pairing with a new partner and cage after isolation, on the hypothesis that the social instability would prolong the recovery of corticosterone levels after the stress of isolation and impede habituation to repeated isolation (McCormick 2010).

The adolescent social instability procedure affected males and females differently over the course of the procedure (e.g., males had reduced weight gain but showed evidence of habituation to the repeated isolation, whereas females did not habituate but weight gain was unaffected), which may be a basis for the greater lasting effect on drug-related behaviour in females. This model of adolescent social stress adds to growing evidence that adolescence is a sensitive period of development during which social experiences can shape the trajectory of ongoing brain development and thereby confer risk or resilience in adulthood (McCormick 2010) since the effect of stress during adolescence also appears to produce changes to hippocampal neurogenesis that last into adulthood (Hueston, Cryan and Nolan 2017).

Individual variability in the social behaviour response to stress e resilience vs. vulnerability

What is the difference between responders and non-responders, or a resilient vs. vulnerable trajectory?

Chronic stress leads to the development of depression– or anxiety–like behaviours in only a subset of laboratory animals (Taliaz et al. 2011, Krishnan et al. 2007, Lehmann and Herkenham 2011, Delgado y Palacios et al. 2011, Golden et al. 2011). Animals which have been termed “resilient” usually exhibit some deleterious symptoms in response to the stress, but do not exhibit deficits in key behavioural domains. For example, following chronic social defeat stress, all genetically inbred C57BL6/J male mice exhibit a constellation of symptoms including heightened reactivity of the HPA axis, deficits in exploratory–based behaviour that are interpreted as increased anxiety, social avoidance and stress–induced polydipsia (Krishnan et al. 2007). However, ~35% of the stressed mice, considered “resilient,” do not exhibit social avoidance, hyperthermia elicited by social interactions, anhedonia–like symptoms (reduced interest in sucrose, high fat food, or sex), or a metabolic syndrome characterized by decreased body weight (Krishnan et al. 2007). Using this classification, resilient animals are not devoid of symptoms and, in fact, exhibit some behavioural adaptations that appear maladaptive, but they exhibit clear resistance to many other maladaptive sequelae of the chronic social stress (Russo et al. 2012).

Several neurotransmission systems are implicated in social stress resilience vs. vulnerability: stress-susceptibility has been correlated with stress-induced increase in levels of brain derived neurotrophic factor (BDNF). Investigation of the individual differences between susceptible and unsusceptible mice revealed that susceptibility was characterized by increased nucleus accumbens (NAc) BDNF levels, but reinforced the importance of BDNF release from the VTA,

as knockdown in the VTA but not NAc promoted resilience (Krishnan et al. 2007). Glutamatergic, serotonergic, and GABAergic systems appear to be involved as well. Vulnerable and resilient animals differ significantly in the expression of AMPA receptors in the dorsal hippocampus, and activation of AMPA receptor during the stress exposure prevented the physiological, neuroendocrine, and behavioural effects of chronic social stress exposure (Schmidt et al. 2010). Knockout of serotonin transporter increases the vulnerability to social avoidance following social defeat (Bartolomucci et al. 2010). Finally, suppression of the GABAergic system is seen in the pre-frontal cortex of mice showing depressive symptoms following social defeat (Veeraiah et al. 2014), and in amygdala of mice exposed to peripubertal stress (Tzanoulinou et al. 2014). Similar suppression is found in the cortex of human patients with PTSD.

Stress impact on memory function

Stress can affect cognition in many ways, with the outcome (i.e., facilitating or impairing) depending on a combination of factors related to both stress and the cognitive function under study (Sandi 2013).

- Stress related factors

In this section we can find 3 essential stress factors that can impact cognitive functions:

1. Source of stress: It refers to the origin of stress with regard to the cognitive task. This factor classifies stress as either *intrinsic* (if stress is originated by elements related to the cognitive task) or *extrinsic* (if stress is originated by conditions completely unrelated to the cognitive task, i.e., in the outside world, and ideally occurring temporally dissociated from such task, i.e., either before or afterwards) (Sandi and Pinelo-Nava 2007).
2. Stressor duration: this factor refers to the length of stress. The differential effects of *acute* versus *chronic* (with some *subchronic* versions) stress have concentrated great interest in the field. In addition to the relevance to cognitive function, this factor is essential when evaluating the neural mechanisms whereby stress affects cognition (Sandi and Pinelo-Nava 2007).
3. Stressor intensity: stressors can vary throughout a very wide range of intensities. Even though oversimplifications can have the drawback of being too superficial, for the sake of clarity, we will just use the categories of *low*, *medium*, *high* (and occasionally *very high*) intensities. Not surprisingly, very high (e.g., a clear life threat, such as a being in a combat)

and mild (e.g., novelty exposure) stressors seem to have distinct effects on cognitive function (Cordero, Merino and Sandi 1998, Joëls et al. 2006).

- Cognitive related factors

The vast majority of research dealing with the cognitive effects of stress has covered quite comprehensively stress actions on different aspects of memory function, including its different phases (acquisition, consolidation, retrieval, etc.), operations (working memory vs long-term memory), types (implicit vs explicit), and strategies (habit vs goal-directed).

Stress and stress mediators appear to exert opposing effects in consolidation (memory storage) and retrieval (access to stored information) (Roozendaal 2002, Roozendaal 2003). If stress is given before learning (acquisition of information), it can potentially affect all cognitive phases involved in memory function. If stress is experienced after learning, any effect observed in retention could now be due to an impact of stress on either consolidation or retrieval, but any effects on acquisition can be discarded. If stress is delivered before the recall test, it should just normally affect the retrieval processes (Figure 2).

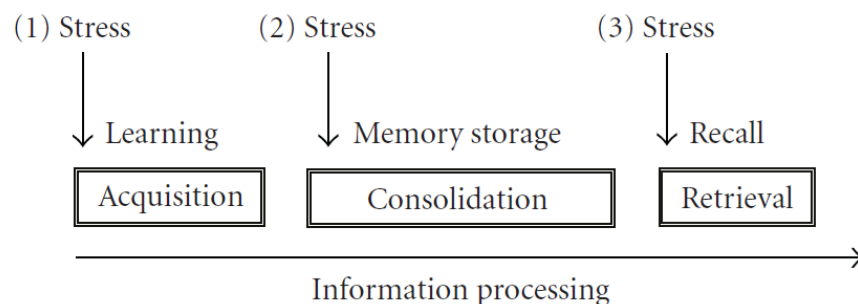


Figure 2. Diagram depicting the relevance of specifying timing of stress with regards to different memory phases.

An additional key factor is the type of the learning process that is evaluated (i.e., implicit/non-declarative learning, explicit/declarative learning, non-associative learning etc.; (Nelson, Schreiber and McEvoy 1992, Squire and Zola 1996, Verfaellie and Keane 1997, Eichenbaum 1999, Moscovitch et al. 2006).

two of probably the most important factors are: psychological factors, like controllability and predictability; and individual differences in the vulnerability and response to stress. The literature indicates the existence of considerable variability in the vulnerability of individuals to display cognitive changes when exposed to stress. Whereas some individuals are particularly ‘vulnerable’, others seem to be quite ‘resistant’ to the effects of stress. These differences could be due to predisposing factors, previous life experiences or, more likely, both. Among the

individual factors, gender (Shors 2004, Andreano and Cahill 2009, McLaughlin, Baran and Conrad 2009), genetic endowment (Palumbo et al. 2010), personality traits (Holmes 2008, Sandi et al. 2008), and age (Bodnoff et al. 1995, Fenoglio, Brunson and Baram 2006) can play an important role in the cognitive consequences of stress.

Chronic stress was originally reported to damage hippocampal structure (McEwen 1999, McEwen 2002), a well-known region in the brain important for memory processing (Eichenbaum 1997). Given the effects of chronic stress in the hippocampus it has been hypothesized that chronic stress affects hippocampal-dependent learning. Chronically stressed male rats were shown to exhibit learning and memory deficits in a variety of spatial tasks, including the radial-arm maze (Luine et al. 1994), the Y-maze (Conrad et al. 1996), and the Morris water maze (Venero et al. 2002, Sandi et al. 2003).

In the hippocampus, chronically activating the stress response can produce maladaptive changes, which have been postulated to contribute to disease (de Kloet et al. 2006, McEwen and Wingfield 2003, Smith 1996). A transition into maladaptive changes includes dendritic remodelling resulting in reduces dendritic arbours in CA3 neurons; dendritic retraction has been observed in other brain regions following 10 to 21 days of repeated stress. When chronic stress continues for 4 weeks, CA1 and dentate gyrus neurons in the hippocampus show dendritic retraction (Sousa et al. 2000). Prefrontal cortical neurons also show dendritic retraction following 1 to 3 weeks of stress (Brown, Henning and Wellman 2005, Radley et al. 2004). Chronic stress-induced CA3 dendritic remodelling has been proposed to be a maladaptive response because it is associated with susceptibility to damage and cognitive dysfunction (McEwen 2016).

Although the majority of studies on chronic stress have focused on structural changes within the hippocampus, chronic stress has opposite effects in the basolateral nucleus of the amygdala (BLA), where it increases the dendritic complexity of neurons (Vyas et al. 2002, Vyas, Pillai and Chattarji 2004), suggesting that chronic stress facilitates memory under emotionally arousing situations.

Can a diet modulate social-stress induced effects?

Food intake is a fundamental basis for survival in all living organisms insofar as an adequate daily food intake secures energy levels (Wolever and Jenkins 1986). Each meal contains a different macronutrient composition that in turn influences a variety of biochemical processes (Wurtman et al. 2003, Gailliot et al. 2007). In addition to supplying the body with nutrients, these biochemical processes also influence brain performance, including higher-level cognition, such as social decision making (Crockett et al. 2008, Colzato et al. 2013, Strang et al. 2017). Therefore, it is not only whether and when we eat that is important, but equally what we eat.

Polyunsaturated fatty acids (PUFAs)

Lipids represent 33%–40% of the energy intake in the United States (Simopoulos 2011).
There are three families of fatty acids classified according to the number of double bonds on their carbon chain they contain: the saturated ones (no double bond), the monounsaturated ones (one double bond MUFA), and the polyunsaturated ones (two or more double bonds, PUFA). In rodents, the brain contains 36%–46% saturated fatty acids, 18%–33% MUFA and 18%–28% PUFAs (Joffre et al. 2016).
PUFAs are classified into two main series, the ω-6 PUFAs and the ω-3 PUFAs depending on the position of the first double bond from the methyl terminal end.
Linoleic acid (LA; 18:2n-6) is the dietary-essential shorter-chain ω-6 PUFA precursor of arachidonic acid (AA), whereas α-linolenic acid (ALA; 18:3n-3) is the precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). AA, DHA, and EPA are also found in the diet, although as distinct sources (Layé et al. 2018).
In the western diet, there is thought to be an imbalance between ω-6 and ω-3 PUFAs, leading to a ω-3 PUFA consumption 12–20 times lower than ω-6 PUFA consumption (Simopoulos 2002, Simopoulos 2011), whereas an optimal ratio should approximately 1(ω-3):3(ω-6). This is due to the increased industrialization in the developed nations accompanied by changes in dietary habits. It is particularly characterized by an increase in LA, abundant in many vegetable oils (60%–65% in sunflower oil for example) (Orsavova et al. 2015) and AA, found in meats (5%–10%) and eggs (15%) (Taber, Chiu and Whelan 1998, Meyer et al. 2003), together with relatively low intakes of ALA, found in some green vegetables, rapeseed oil (10%) (Lewinska et al. 2015), and nuts, and EPA and DHA abundant in fatty fish (18.7% EPA plus DHA in salmon, 32.9% EPA plus DHA in tuna) (Strobel, Jahreis and Kuhnt 2012).

PUFAs from the diet are absorbed from the gut to the blood and are available for storage (in the adipose tissue), conversion into longer-chain PUFA (mainly in the liver), or energy production through β-oxidation. LA and ALA biosynthetic pathway to AA and EPA and DHA, respectively, involves a series of desaturation, elongation occurring and β-oxidation (Layé et al. 2018) (Figure 3).

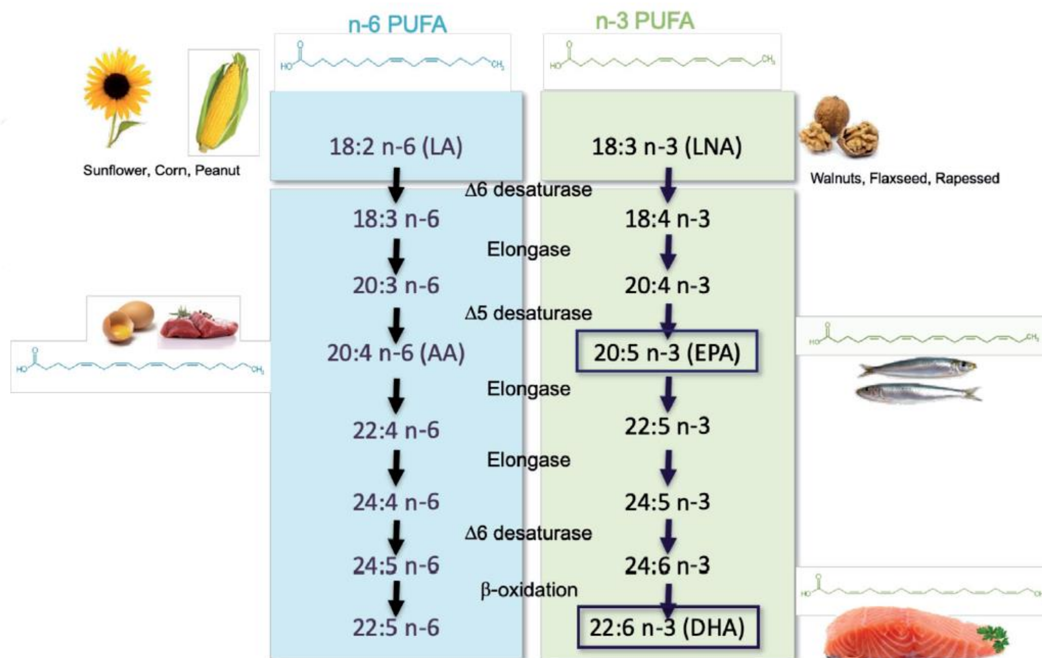


Figure 1. Synthesis pathways of n-6 and n-3 LC-PUFA and main dietary sources of PUFAs. LA: linoleic acid; *LNA:* linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid. (Joffre 2019)

Brain Omega-3 PUFAs

The brain contains high levels of PUFAs (25–30%) that are mainly DHA, (12–14% of total fatty acids) and AA, (8–10% of total fatty acids) (Carrié et al. 2000, Xiao, Huang and Chen 2005, McNamara and Carlson 2006, Chung, Chen and Su 2008, Joffre, Rey and Layé 2019).

EPA level is low in the brain because of its rapid β-oxidation, elongation and desaturation to docosapentaenoic acid (DPA n3; 22:5n-3) and DHA, and is not heavily recycled within brain phospholipids (Chen and Bazinet 2015).

Saturated and MUFAs can be synthesised de novo within the brain, but PUFAs are mainly supplied by the blood (Bourre et al. 1984).

The brain expresses the enzymes that are necessary for the synthesis of DHA and ARA. However, in rodents the synthesis rate of these PUFAs in the brain is much lower than the rate of PUFA uptake from the plasma. Furthermore, the brain levels of enzymes involved in the synthesis of ARA and DHA seem to be static, in contrast to the liver, which regulates the expression of these enzymes in response to dietary supply (Rapoport 2013). Upon entry into

the brain, most PUFAs — especially DHA and ARA — are activated by a long-chain-fatty-acid-CoA synthase (ACSL) (Watkins 1997) and then esterified to phospholipid membranes. In phospholipids, two fatty acids attach to the stereospecifically numbered first and second carbons (sn-1 and sn-2 positions) of the glycerol molecule. Upon esterification to the phospholipid plasma membrane, fatty acids at the sn-1 position can be de-esterified and released from the membrane by phospholipase A1 (PLA1), whereas fatty acids at the sn-2 position (such as ARA and DHA) are de-esterified by phospholipase A2 PLA2 (Burke and Dennis 2009).
PUFAs' concentration vary across brain regions (Delion et al. 1994, Carrié et al. 2000, McNamara et al. 2009, Joffre et al. 2016). For example, in the adult C57BL6/J mice, the highest level of AA is found in the hippocampus (10.2%), followed by the prefrontal cortex (9.7%), the hypothalamus (8.5%), the cortex (7.7%), the cerebellum (6.5%), and the brain stem (5.5%) (Joffre et al., 2016). The highest level of DHA is found in the prefrontal cortex (14.3%) and in the hippocampus (13.7%), followed by cerebellum (12.2%), cortex (11.9%), hypothalamus (10.1%), and brain stem (8.2%) (Joffre et al. 2016).
This brain fatty acid composition can be affected by environmental factors such as nutrition .Indeed, PUFA content in all brain structures is strongly impacted by PUFAs present in the diet (Alashmali, Hopperton and Bazinet 2016).
A diet rich in ω-3 PUFAs increases brain DHA in rodents (Hiratsuka et al. 2009, de Theije et al. 2015, Skorve et al. 2015, Kitson et al. 2016). However, DHA supplementation is more effective than α-linolenic supplementation in increasing the DHA content in the brain (Lacombe et al. 2017, Rey et al. 2019). In rodents, DHA supplementation from 16 weeks to 16 months or from 20 to 22 months of age compensates a DHA decrease due to aging (Labrousse et al. 2012, Bascoul-Colombo et al. 2016). On the other hand, a diet deficient in ω-3 PUFAs induces a decrease in brain DHA levels (Connor, Neuringer and Lin 1990, Carrié et al. 2000, Larrieu et al. 2012, Joffre et al. 2016) and an increase in brain DPA (22:5n-6) and often AA levels (Connor et al. 1990, Larrieu et al. 2012). These modifications impact all brain structures, but some of them are more affected than others: the prefrontal cortex and the hippocampus, which contain the highest DHA levels are the most sensitive, whereas the hypothalamus, which contains the lowest DHA, is the least sensitive. These differences may be attributed to the evolution of brain structures, hence capability (Crawford et al. 1999, Broadhurst et al. 2002).
Both, AA and DHA (and their metabolites), participate in many important brain functions such as acting as intracellular second messengers, neurotransmission, gene transcription, among other brain processes (Hibbeln, Palmer and Davis 1989). Additionally, fatty acids at the cell membrane can directly interact with membrane proteins, determining their structure and

function. Thus, they can determine the membranes fluidity, lateral pressure, bilayer thickness, and surface charge distribution (Stillwell and Wassall 2003).

Therefore, the presence of DHA in the membrane can modulate neurotransmission systems signalling, contributing in this way to brain function. PUFAs and/or their mediators are agonists for the oxysterols receptor LXR, peroxisome proliferator-activated receptor (PPAR), hepatic nuclear factor 4A (HNF4A; also known as NR2A1), chemokine-like receptor 1 (also known as CHEMR23), G-protein-coupled receptor 32 (GPR32) and lipoxin receptor ALX/ FPR2, and they can activate protein kinase C (PKC) and inhibit nuclear factor-κB (NF-κB) (Green, Orr and Bazinet 2008, Rapoport 2014, Serhan 2014, Rao et al. 2008).

PUFAs can also influence brain function through modulation of the endocannabinoid system (Bazinet and Layé 2014); endocannabinoids are important regulators of synaptic function; they suppress neurotransmitter release (including the release of glutamate, GABA, monoamine neurotransmitters, opioids and acetylcholine) by acting as retrograde messengers at presynaptic CB1s (Castillo et al. 2012).

Neuroinflammation

Inflammation is a key mechanism in the pathophysiology of mood disorders, including major depression, post-partum depression and bipolar disorder (Dantzer et al. 2008, Capuron and Miller 2011). Increased levels of inflammatory factors, such as proinflammatory cytokines and chemokines, are found in a subset of depressed patients and may contribute to their symptoms through a direct effect in the brain (Raison and Miller 2011).

ω-3 PUFAs have powerful anti-inflammatory properties (Calder 2005) because they play an important role in the regulation of the synthesis and release of some pro-inflammatory mediators (Delpech et al. 2015, Hanisch and Kettenmann 2007, Cunningham and Sanderson 2008, Yirmiya and Goshen 2011, Pascual et al. 2012).

DHA and EPA effect on neuroinflammatory pathways could be either direct or indirect.

High levels of brain DHA are linked to reduced expression of proinflammatory cytokines (including interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) in several rodent models of acute or chronic neuroinflammation, such as systemic administration of the bacterial endotoxin lipopolysaccharide (LPS), brain ischemia-reperfusion, spinal cord injury, or aging (Orr et al., 2013b). In addition, a diet rich in EPA attenuates the production of the proinflammatory cytokine IL-1β and improves synaptic plasticity impairment in the hippocampus of old rats (Martin et al. 2002, Lynch et al. 2007). Importantly, the reduction

of neuroinflammation linked to diets enriched in ω-3 -PUFA is associated with improvement of spatial memory deficits (Song et al. 2004, Labrousse et al. 2012).

Several mechanisms have been proposed to explain the indirect immunomodulatory properties of ω-3 PUFAs. One of the most attractive is the synthesis of bioactive lipid mediators or oxylipins. These oxylipins are synthesized sequentially: first, those involved in the regulation of inflammation such as the eicosanoids (prostaglandins, leukotrienes, thromboxane), and then those involved in the resolution of inflammation called Specialized Pro-resolving Mediator (SPMs) (resolvins, protectins, maresins; Figure 4). SPMs have both anti-inflammatory and pro-resolutive properties without immune suppression and induce a return to homeostasis (Serhan et al. 2000, Serhan et al. 2002, Serhan, Chiang and Van Dyke 2008a, Serhan 2014). They actively coordinate and finely tune the inflammatory response. They down-regulate the pro-inflammatory cytokines and up-regulate the anti-inflammatory cytokines, promote the phagocytosis of cellular debris and dead cells without immune suppression, reduce the concentration, and compete with pro-inflammatory oxylipins derived from ω-6 PUFAs (Joffre et al. 2020).

PUFAs are released from membrane phospholipids through the action of phospholipases A2 (PLA2) in response to stimulation. DHA is hydrolysed by calcium independent PLA2 (iPLA2) from phospholipids and plasmenylethanolamine-PLA2 from plasmalogens (Farooqui and Horrocks 2006). After this step, ω-3 PUFAs undergo an enzymatic conversion to generate SPMs (ω-3 PUFA-derived SPMs are synthesized mainly from DHA and EPA via COX-2, lipoxygenases (LOX) and CYP450 monoxygenases (CYP450) (Figure 4). In the brain, 15-LOX, 12/15- LOX and 5-LOX are the most abundant LOX and are widely distributed, suggesting the potential production of SPMs (Shalini et al. 2018). They are expressed in the second step of inflammation in the hippocampus (Czapski, Gajkowska and Strosznajder 2010, Birnie et al. 2013). 15-LOX is involved in neurodegeneration and neurotoxicity due to the increased oxidative stress it generates in models of Alzheimer's disease (Praticò et al. 2004, Wang et al. 2015b) and brain ischemia (Yigitkanli et al. 2017). However, it is also implicated in neuroprotection (Sun et al. 2015). Indeed, it increases the production of 12-HETE and 15-HETE that promote the activation of PPARγ that is neuroprotective through its anti-inflammatory properties. The inhibition of 15-LOX induces hippocampus-dependent cognitive alterations (Shalini et al. 2018).

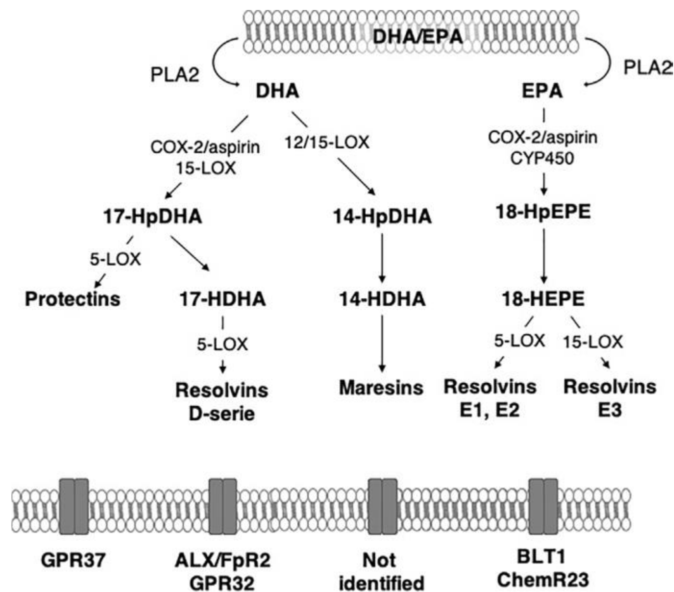


Figure 2. Main synthesis pathway of n-3 long-chain PUFA-derived lipid mediators.
ALX/FpR2, lipoxin A4 receptor/formyl peptide receptor 2; BLT1, Leukotriene B4 receptor 1; ChemR23, chemokine-like receptor 1; COX-2, cyclooxygenase-2; CYP450, monoxygenases cytochrome P450; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GPR, G protein-coupled receptor; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HpDHA, hydroperoxyl-docosahexaenoic acid; HpEPE, hydroperoxy-eicosapentaenoic acid; LOX, lipoxygenases; PLA2, phospholipase A2 (Joffre et al. 2019).

In the brain, the inducible COX-2 is activated *via* an NFκB pathway (Nadjar (Nadjar et al. 2005)et al., 2005). COX-2 catalyses the first step of the synthesis of prostaglandins and thromboxanes derived from ω-6 PUFAs that contribute to the initiation of inflammation (Davidson (Davidson et al. 2001, Salinas et al. 2007, Engström et al. 2012)et al., 2001; Salinas et al., 2007; Engstrom et al., 2012). DHA is the precursor of resolvins D1-6 (RvD1-6), neuroprotectin D1 (NPD1) and maresins 1–2 (Mar1-2) which all have pro-resolutive and anti-inflammatory properties (Spite (Spite and Serhan 2010, Halade, Black and Verma 2018) RvD1-6 are synthesized from DHA but RvD1 is the most studied because it has powerful anti-inflammatory and pro-resolutive properties. DHA is converted into monohydroxy DHA, 17-hydroxy docosahexaenoic acid (17-HDHA) by acetylated COX- 2, CYP450 and 15-LOX (Barden, Mas and Mori 2016, Halade et al. 2018) and then into RvD1 by 5-LOX (Sun et al. 2007, Recchiuti 2013). DHA is also converted into di-hydroxy-DHA, termed protectin D1 (PD1) or neuroprotectin D1 (NPD1) when produced in the central nervous system by 5- and 15-LOX (Hong et al. 2003, Aursnes et al. 2014, Kuda 2017, Doyle, Sadlier and Godson 2018). Acetylated COX-2 permits the synthesis of aspirin-triggered PD1 (AT-PD1) which has powerful protective effects (Bazan et al. 2012). DHA is transformed into Mar 1-2 by 12/15-LOX *via* the synthesis of 14-HDHA (Serhan et al. 2008b, Barden et al. 2016, Halade et al. 2018).

EPA is the precursor of resolvins E1 (RvE1), E2 and E3 that have many biological roles (Serhan et al. 2000, Rey et al. 2016, Halade et al. 2018). It is converted by aspirin-triggered acetylated COX-2 or CYP450 into 18R-hydroxyeicosapentaenoic acid (18R-HEPE), that is transformed into RvE1 or E2 by 5-LOX (Ohira et al. 2010, Barden et al. 2016) or into RvE3 by 15-LOX (Isobe et al. 2012).

RvD1 and RvE1 display anti-inflammatory activities in the CNS (Joffre et al. 2020). Indeed, an intrathecal injection of 17-HDHA, precursor of RvD1, decreases TNF-α release in the spinal cord in rats (Abdelmoaty et al. 2013) and the expression of hippocampal pro-inflammatory cytokines IL-1β and TNF-α induced by LPS acute icv injection. Moreover,17-HDHA restores transmission and synaptic plasticity and prevents astrogliosis and cognitive decline in a systemic inflammation model in mice (Terrando et al. 2013). RvE1 reduces the expression of pro-inflammatory cytokines IL-1β and IL-6 in the prefrontal cortex (Kantarci et al. 2018).

Cognition

Animal studies using diets that are lacking or are enriched in ω-3 PUFAs have indicated that there is a critical period for DHA accretion in the brain, normal brain development and cognition. (Bazinet and Layé 2014).

Rodents with lower brain DHA levels show decreased performance in learning tasks (Moranis et al. 2012, Fedorova et al. 2007). On the other hand, chronic ω-3 PUFA supplementation improved long-term memory and increased synaptic plasticity in the hippocampus of stressed and aged rodents (Joffre et al. 2014). Other studies showed that dietary supplementation with ω-3 PUFAs facilitated LTP in reodents' hippocampus (Connor et al. 2012, Kavraal et al. 2012).

Dietary enrichment of aged rats with EPA, DHA have all been shown to have positive effects on age-related impairments in LTP, and these effects are likely mediated via multiple anti-inflammatory effects acting via alterations in cytokine levels. Feeding rats diets supplemented with EPA prevented age-related increased in cortical and hippocampal IL-1β and restored LTP (Martin et al. 2002), it also prevented age-related increases in IL-1 β-induced signalling and decrease in IL-4, extracellular-signal-regulated kinases(ERK) and PI-3 kinase (Maher, Martin and Lynch 2004). EPA also protects aged rats from amyloid- β (A β) induced increases in hippocampal IL-1β, potentially mediated by positive effects on the PPARγ nuclear transcription factor (Minogue et al. 2007). In mouse models of Alzheimer's disease, increased brain DHA levels are associated with a reduction in the formation of amyloid plaques (Calon et al. 2004,

Lim et al. 2005, Green et al. 2007), a protective effect that could be due to the anti-amyloidogenic activity of DHA or of its mediator NPD1 (Zhao et al. 2011, Calon et al. 2005).

Mood and stress

Findings from clinical and observational studies suggest that PUFAs have a role in the regulation of mood. EPA concentration (Adams et al. 1996, Green et al. 2006, Liu et al. 2013) as well as DHA concentration (Edwards et al. 1998, Frasure-Smith, Lespérance and Julien 2004, Green et al. 2006, McNamara and Liu 2011, Liu et al. 2013, Otoki et al. 2017) are decreased in the membrane of erythrocytes and in the plasma of patients suffering from unipolar depression, seasonal winter affective disorder or social anxiety disorders (Adams et al. 1996, Green et al. 2006). The involvement of brain DHA levels in the development of depression has also been assessed in animal studies. Single- or multi-generation exposure to dietary ω-3 PUFA deprivation induces depressive and anxiety-like behaviours in rats and mice (Lafourcade et al. 2011, Rao et al. 2007, DeMar et al. 2006), and these behaviours are associated with decreased brain DHA levels, including in the prefrontal cortex and the hippocampus (Lafourcade et al. 2011).

Stress may be one pathway by which ω-3 PUFA levels modulate mood and cognition. Stress and depression, as well as dietary composition akin to the Western diet with high ω-6 to ω-3 PUFA ratio, have been shown to influence inflammation through increasing pro-inflammatory cytokine production (Kiecolt-Glaser 2010, O'Brien, Scott and Dinan 2004). Animal studies suggest that ω-3 PUFAs mitigate stress-induced cognitive impairments (Su 2010). Further, an ω-3 PUFA deficient diet is associated with learning deficits and heightened anxiety (Heinrichs 2010), whereas ω-3 PUFA supplementation in rats prevented anxiety- and depressive-like behaviours and learning and memory deficits induced by stress (Ferraz et al. 2011). Some studies show that behavioural impairments (e.g., anxiety-like behaviour and social interaction) occur in mice after exposure to chronic social defeat stress (CSDS; (Golden et al. 2011, Bosch-Bouju et al. 2016, Larrieu et al. 2017). By comparing the effects of dietary ω-3 PUFAs deficiency to those of CSDS on emotional behaviour, Larrieu and collaborator found that mice fed with a diet deficient in ω-3 PUFAs exhibited behavioural changes and neuronal atrophy profile that resemble those of mice exposed to CSDS (Larrieu et al. 2014). Interestingly, behavioural alterations can be reversed after chronic ω-3 PUFAs supplementation. As such, increased anxiety- and depressive-like behaviour after chronic stress is normalized after ω-3 PUFAs supplementation (Ferraz et al. 2011, Larrieu et al. 2014).

Vitamin A

Vitamin A (all-trans-retinol) is a fat-soluble micronutrient which, together with its natural derivatives and synthetic analogues that exhibit its biological activity, constitutes the group of retinoids (McLaren and Kraemer 2012). It is converted by two successive oxidative reactions into its main biologically active derivatives, retinaldehyde and retinoic acid (RA). Vitamin A is the most multifunctional vitamin in the human body, as it is involved in several essential physiological processes from embryogenesis to adulthood. Most of these functions are not carried out by retinol itself but by its active metabolites (Timoneda et al. 2018) (Figure 5).

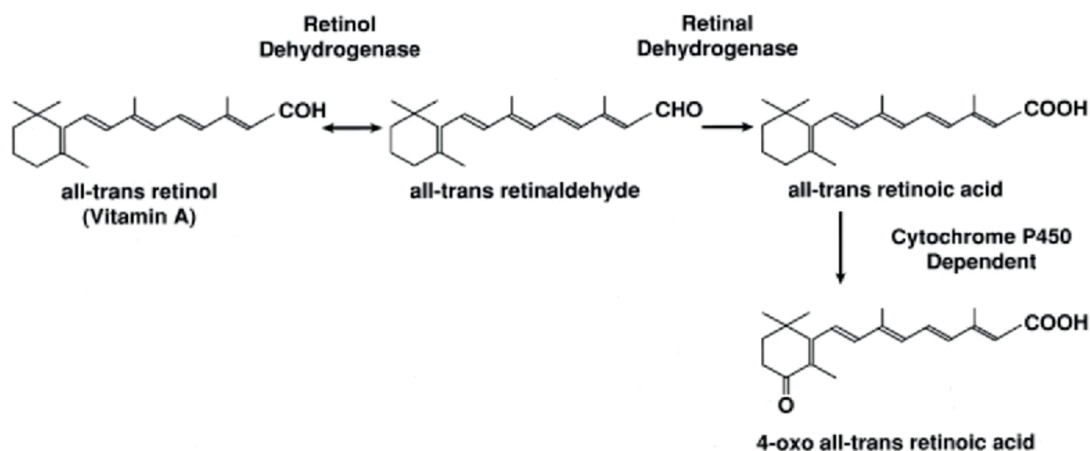


Figure 3. Intracellular metabolism of retinol (vitamin A). All-trans retinol (vitamin A) is converted to all-trans retinaldehyde by retinol dehydrogenase. The second and irreversible step is the oxidation of retinaldehyde to all-trans-retinoic acid by retinal dehydrogenase 1 family. Excessive all trans-retinoic acid is not recycled back to retinol and must be oxidized to be eliminated from the body by the cytochrome P450 family members in 4-oxo all-trans retinoic acid.

No animal species have the potential for *de novo* synthesis of vitamin A. Hence vitamin A must be acquired from the diet as preformed retinol or retinyl esters or as precursors, the carotenoids, mainly β-carotene. Diet retinol is provided by eggs and liver from several animals, especially poultry and fish (Fraser and Bramley 2004). Dietary carotenoids are provided by fruits and vegetables with orange/yellow pigments and then converted to retinol by a number of tissues in the body.

Retinol is stored as retinyl esters essentially in the liver (O'Byrne and Blaner 2013, Shirakami et al. 2012). Mobilisation of retinol from its stores requires hydrolysis of retinyl esters and secretion of retinol to the circulation. As it is poorly soluble in water, retinol circulates in blood bound to a soluble carrier protein termed serum retinol-binding protein (RBP) that is associated with another protein named transthyretin (transporter of thyroxin and retinol, TTR) (Noy and Xu 1990) (Figure 5). Then the major fraction of cellular uptake of retinol from circulation

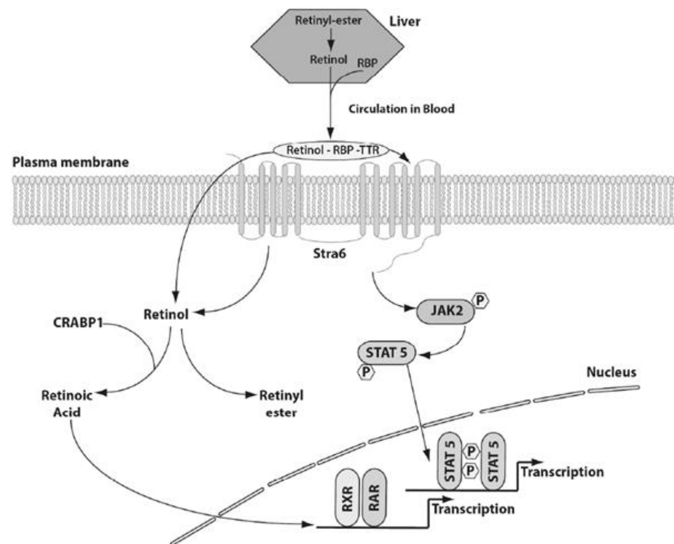
occurs upon dissociation from RBP and free diffusion across the plasma membrane (Noy 1992). However, in tissues with extreme vitamin A demand, such as retinal pigment epithelium cells in the eye, a transporter has been identified. This transporter named Stra6 is an integral plasma membrane protein, which recognizes circulating retinol-RBP and mediates retinol uptake into cells (Kawaguchi et al. 2007, Ruiz et al. 2012). It is important to consider that a number of factors can affect the vitamin A absorption and availability and thus its requirements, including the presence and severity of infection and parasites, intestinal or liver disease (such as biliary atresia, cholangitis, viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease), iron and zinc status, stress, fat intake, xenobiotics, protein energy malnutrition, alcohol consumption and the food matrix and food processing. Both insufficient dietary retinoid intake (hypovitaminosis A or vitamin A deficiency, VAD) and excessive retinoid consumption resulting in vitamin A concentrations above the physiological range (hypervitaminosis A or vitamin A-toxicity) cause adverse effects to human health, which are paradoxically similar in both situations (Blaner et al. 2016, Lieber 2000, World Health 2009, Eroglu and Harrison 2013, Otten, Hellwig and Meyers 2006, Shmarakov 2015).

Retinoids and its natural derivatives retinol, retinal and RA, are involved in many important physiological functions, such as vision, immunity, reproduction, embryonic development, cellular differentiation, tissue architecture maintenance, antioxidant function, redox signalling or energy balance (De Luca 1991, Livrea and Tesoriere 1998, Clagett-Dame and Knutson 2011, Rhinn and Dollé 2012, Ross 2012, Sommer and Vyas 2012, Al Tanoury, Piskunov and Rochette-Egly 2013, Baybutt and Molteni 2007). Retinal, the oxidized form of retinol, plays a key role in vision being the precursor of the visual chromophore 11-cis-retinal. The photosensitive receptor is restored via the retinoid visual cycle (Blomhoff and Blomhoff 2006, Wald 1968). RA is involved in the regulation of more than 500 genes. In some cases, the control of gene expression is exerted by RARs directly, mainly by direct binding of RAR/RXR heterodimers to RA responding elements (RAREs) on the promoter of responsive genes. However, in some cases, gene regulation is achieved through an indirect action of RARs onto responsive genes (Figure 5). At the moment, two families of nuclear receptors, RA receptors (RAR isotypes α, β and γ and their isoforms) and retinoid X receptors (RXR isotypes α, β and γ and their isoforms) are described. RARs act by forming heterodimers with RXRs, whereas RXRs can form either homodimers or heterodimers with several partners including RARs, vitamin D receptor, PPAR, thyroid hormone receptor and orphan nuclear receptor (Timoneda et al. 2018).

Several studies over the last decade have suggested that RA displays biological activities that are independent of its ability to activate RAR. RA can also function as an agonist for a different nuclear receptor, namely PPARβ/δ. PPARs, like RARs, interact with RXR to form heterodimers which when are activated by its ligand bind to PPAR response elements, PPRE, in regulatory regions of specific genes to induce target gene transcription. PPARβ/δ is involved in keratinocyte differentiation, neuronal development and inflammation and, like other PPARs, is also involved in lipid metabolism and insulin resistance. RA signalling through RXR: PPARβ/δ has acquired a great interest for energy homeostasis and insulin response (Berry and Noy 2009, Noy 2016).

Retinol can also work as a cytokine that activates Stra6 and transduces a signalling cascade mediated by tyrosine kinases called Janus kinases (JAK) and by their associated transcription factors STAT (Signal Transducers and Activators of Transcription) (Figure 6). Upon dimerization, STATs translocate into the nucleus where they regulate the expression of target genes involved in energy homeostasis and insulin responses (Iskakova et al. 2015).

The importance of vitamin A for mammals was first evident with the discovery that vitamin A is required for the function of the eye (Wald 1935). A lack of vitamin A leads to decline and loss of vision, as this vitamin has a fundamental role in retinal function and visual transduction. The earliest sign of vitamin A deficiency is xerophthalmia, which can start with nightblindness, reflecting a decline in the retina’s capacity for light detection (Sommer 1998). Nightblindness is reversible with restoration of vitamin A, but the vitamin is also necessary for the health of the cornea, the transparent structure in the front of the eye. Later stages of xerophthalmia resulting from vitamin A deficiency can include ulceration of the cornea and finally corneal melting, causing irreversible blindness (Sommer 1998). Vitamin A is even necessary for the development of the eye in the foetus, and vitamin A deficiency can cause severe malformation of this structure (See and Clagett-Dame 2009).



<u>Figure 4</u>. Signalling by vitamin A (Retinol): Retinol circulates in the blood bound to RBP-TTR. Retinol uptake into the cell occurs in majority by passive diffusion across the membrane. In the eye, it involves a transporter Stra6. Then in the cell, retinol binds CRBP1, which controls its partitioning between metabolism or esterification for storage. Retinol bound to RBB can also activate Stra6 that initiate a signalling cascade leading to the activation of STAT5 that translocate in the nucleus and regulate the expression of a subset of target genes (Iskakova et al. 2015).

Brain Vitamin A

The brain's requirement for vitamin A is not driven by a need for the vitamin per se, but instead for vitamin A-derived retinoic acid that is generated in two steps by enzymes [retinol dehydrogenases and retinaldehyde dehydrogenases (RALDHs)] present in those cells that activate vitamin A (Napoli 2012). These are the two main routes by which vitamin A acts in the brain, while the system is turned off by breakdown of retinoic acid by a set of enzymes known as CYP26s. The functions of vitamin A and retinoic acid in controlling cell proliferation and differentiation had previously suggested only minor functions for this vitamin in the brain, given that neurons are non-proliferative. However, vitamin A is essential for neuroplasticity that allows neurons to alter their connections with other cells and the strength of the signal passed among them (Stoney and McCaffery 2016).

However, evidence from studies of animal models suggests that vitamin A is essential for a number of key functions of the brain (Shearer et al. 2012), and these may be affected by even relatively mild vitamin A deficiency or can be problematic when combined with a genetic susceptibility to vitamin A deficiency, such as in individuals who cannot make efficient use of carotenes (Leung et al. 2009).

Neuroinflammation

Retinoids have important roles in prevention of neuroinflammatory responses for providing neuroprotection (Lee et al. 2009). Retinoids are known to down regulate expression of cytokines and inflammatory molecules in microglia (Goncalves et al. 2013).

Inflammation causes synaptic dysfunction and neurodegeneration in the brain. Production of excessive neuroinflammatory mediators. Optimal microglial function is necessary for scavenging tasks, but chronic activation of these cells in the brain also causes proinflammatory responses, oxidative stress, degradation of neuroprotective retinoids, and down regulation of RA signalling, promoting degeneration of surrounding healthy neurons (Regen et al. 2017).

In the context of neuroinflammation RA was shown to mitigate the activity of microglia cells (Choi et al. 2009, Dheen et al. 2005, Xu and Drew 2006). Most pathological features associated with microglial reactions involve the activation of astrocytes, which are influenced by RA as well. The RXR agonist 9-cis RA suppressed the production of TNFα that was triggered by LPS in astrocyte primary cultures (Xu and Drew 2006). Stimulation with bacterial LPS induced mouse cortical astrocytes to release cytokines IL-1β, IL-6, IL-12, and TNFα. These factors are involved in cellular degeneration under neuroinflammatory conditions which are associated with pathologies such as ischemia, CNS injury and neurodegenerative disorders, such as Alzheimer’s disease (Weisman, Hakimian and Ho 2006). Pretreatment of astrocyte primary cultures with all-trans RA interfered with the LPS-induced mRNA expression and protein release of these cytokines (Das, Dasgupta and Ray 2019).

RXRs are known to heterodimerize also with other members of the nuclear hormone receptor superfamily, including peroxisome proliferator-activated receptors (PPAR), vitamin D receptor, thyroid hormone receptor (TR), or liver X receptor (LXR). Since several of these nuclear receptors were shown to have anti-inflammatory properties themselves, the RXR family is in a pivotal position for the development of therapeutic approaches (Zhang-Gandhi and Drew 2007, Genovese et al. 2005).

Cognition

The effects of the vitamin A on regions of the central nervous system include the brain. The first clear evidence that retinoids play a role in cognitive function came from work with knockout mice that lacked either one of the retinoic acid receptors, RARβ, or one of the retinoid × receptors, RXRγ (Chiang et al. 1998). These particular receptors are uniquely expressed in hippocampal regions of the adult mouse brain that are implicated in spatial and relational memory, whereas the other retinoic acid receptors and retinoid × receptors are more uniform in

their distribution. Thus, mutant mice for these receptors showed normal development and growth with no abnormal physical or neuronal morphology yet demonstrated cognitive deficits in learning the Morris water maze and impaired motor control and balance, compared to wild-type mice. These behavioural impairments correlated with electrophysiological differences in hippocampal CA1 cells in that RARβ mutants had impaired long-term potentiation (LTP) and the RARβ and RXRγ mutants both impaired LTD. Both of these correspond to changes in long-term synaptic efficacy that can affect learning and memory. Following these findings, several studies have demonstrated a dietary link between retinoids and behaviour or neuronal plasticity. By experimentally inducing Vitamin A Deficency (VAD) in neonatal mice, Misner *et al.* showed that poor retinoid nutrition also affects LTP and LDP in mice, along with the more obvious physical and ocular deformities typical of VAD (Luo, Wagner and Dräger 2009). These electrophysiological effects occurred without apparent physical differences in the underlying neuronal structure of the hippocampus, and in fact when proper retinoid nutrition was returned to retinoid-deprived mice, LTP and LTD returned to normal (Luo et al. 2009). Behaviourally, dietary VAD in rodents also results in cognitive declines in memory tasks, but unlike the apparent rescue effect of supplemental retinoids for electrophysiological function, the behavioural rescue effect was not as consistent in all animals as there continue to be age-related effects on susceptibility to VAD (Etchamendy et al. 2003).

In parallel to the deprivation experiments, high doses of the 13-*cis* retinoic acid isomer administered to adult mice also resulted in cognitive deficits and were correlated with reduced cell proliferation in the hippocampus and the proliferative regions of the ventricle (Crandall et al. 2004). Thus, excessively high levels of retinoic acid also have detrimental effects, suggesting that it needs to be regulated within a narrow concentration range. Furthermore, in a mouse model of Alzheimer's disease that overexpresses genes for β-amyloid and presenilin 1, mice can be rescued from Alzheimer's-related learning deficits by therapeutic all-*trans* retinoic acid (ATRA) administration (Ding et al. 2008).ATRA-treated mice showed fewer of the neurodegenerative β-amyloid deposits in their brains, yet the possibility exists that the cognitive improvements were unrelated to the decrease in β-amyloid deposits, as aged wild-type mice that are given retinoic acid also show improvements in their cognitive abilities (Etchamendy et al. 2001). Indeed, retinoid based therapeutic approaches are promising for nervous system injuries, age-related declines in cognitive function as well as dementia-associated diseases; however, because of the multiple gene/signalling pathways and multiple aspects of neuronal plasticity known to be affected by retinoid signalling, further research is needed (Olson and Mello 2010).

Interaction between Omega-3 PUFAs and vitamin A

Retinoids and ω-3 PUFAs may modulate cerebral plasticity and memory by regulating gene expression through nuclear receptors that function as ligand-controlled transcription factors (Lane and Bailey 2005, Su 2010). Indeed, DHA and RA can bind to nuclear receptors, such as the PPARs, the RARs, and the RXRs (Evans and Mangelsdorf 2014). Several studies highlighted multiple levels of interactions between the fatty acid and the retinoid signalling pathways. On the one hand, it has been shown that RXR is the obligatory heterodimerization partner of RARs and PPARs, suggesting that RXRs play a key role in both retinoid- and ω-3 PUFA-mediated signalling pathways (van Neerven, Kampmann and Mey 2008). On the other hand, in vitro studies have shown that fatty acids and particularly DHA can bind and activate RXRs (de Urquiza et al. 2000, Lengqvist et al. 2004) and that RA can bind to the PPARs (Shaw, Elholm and Noy 2003, Schug et al. 2007), implying interactions at the nuclear level between DHA and RA for binding to their receptors. Moreover, RA and ω-3 PUFAs have additional extra- nuclear and non-transcriptional effects that activate kinase signalling pathways such as, AKT or the MAPK, which includes ERK1/2, thus influencing gene expression through phosphorylation processes (Masiá et al. 2007, Al Tanoury et al. 2013). These signalling pathways are involved in the modulation of cerebral plasticity and thus in learning and memory processes (Giese and Mizuno 2013). It has been shown that the ERK2 mRNA expression is impaired in the rat hippocampus during aging (Simonyi, Murch and Sun 2003). Other kinases such as the CAMKII involved in synaptic plasticity (Ma, Li and Tsien 2015) seem to be modulated by both ω-3 PUFAs and RA. Indeed, although the transcriptional regulation of CAMKII depends on retinoids (Chen and Kelly 1996), it has been shown that DHA treatment normalizes the CAMKII expression in the hippocampus of rats after a traumatic brain injury (Wu, Ying and Gomez-Pinilla 2011).

A recent work of Létondor and collaborators demonstrated for the first time a preventive additive effect of an EPA/DHA and vitamin A enriched diet on the age-related decline in reference memory performance that could be in part mediated both by RXRγ and kinase signalling pathways that were maintained in the hippocampus of middle-aged supplemented rats (Létondor et al. 2016).

Altogether these data indicate that there is a close relationship between the ω-3 PUFA and the retinoid signalling pathways with both intra-and extra-nuclear interactions, suggesting that these nutrients may act together to modulate synaptic plasticity processes and memory.

Fatty acids 'derivatives: Oleoylethanolamide

Fatty acid ethanolamides (FAEs) exist as a class of lipid amides that regulate numerous pathophysiological functions. This class includes the FAEs palmitoylethanolamide (PEA), oleoylethanolamine (OEA), and linoleoylethanolamide (LEA) that possess a variety of physiological activities (Calignano et al. 1998, Rodríguez de Fonseca et al. 2001, Lo Verme et al. 2005, LoVerme et al. 2006, Fu et al. 2003).

OEA is formed from the ω-9 monounsaturated fatty acid, oleic acid. The identified biological functions of OEA include promotion of fat catabolism and control of food intake (Lo Verme et al. 2005, Thabuis et al. 2007, Thabuis et al. 2008, Piomelli 2013), primarily through activation of the nuclear transcription factor peroxisome proliferator-activated receptor α (PPAR-α) (Fu et al. 2003). Diets high in oleic acid may beneficially modify body composition and regional fat distribution (Walker et al. 1996, Paniagua et al. 2007, Gillingham, Robinson and Jones 2012, Estruch et al. 2016). Emerging evidence suggests OEA may mediate this response (Pu et al. 2016), positioning OEA as an attractive molecule in the current obesogenic environment that requires further exploration.

OEA has also attracted attention as a lipid mediator involved in peripheral appetite regulation. In rodents, intestinal OEA levels decrease during starvation (Rodríguez de Fonseca et al. 2001, Fu et al. 2007) and both intraperitoneal injection as well as oral administration of OEA decrease food intake (Rodríguez de Fonseca et al. 2001, Nielsen et al. 2004, Oveisi et al. 2004). OEA stimulates hepatic lipolysis, decreases body weight gain and lowers hepatic and adipose tissue hyperlipidemia in obese rats probably through activation of the nuclear receptor PPAR-α (Fu et al. 2003, Fu et al. 2005, Yang et al. 2007). Levels of OEA in plasma seem to be influenced by glucose levels (Matias et al. 2007). OEA is also an endogenous ligand for GPR119 (Overton et al. 2006) which is expressed primarily in the pancreas and to some extend in the small intestine (Soga et al. 2005, Sakamoto et al. 2006). Activation of GPR119 leads to reduction in both food intake, bodyweight gain and white adipose deposition in diet-induced obese rats (Overton et al. 2006) and it improves glycemic control by enhancing glucose-dependent insulin release (Chu et al. 2007). Moreover, OEA is an endogenous ligand for the pain-mediating transient receptor potential vanilloid 1 (Movahed et al. 2005, Zygmunt et al. 1999).

OEA inhibits food intake mainly, but not essentially by recruiting sensory afferents in the intestinal branch of the vagus nerve (Azari et al. 2014). Several studies demonstrated this mechanism of action: (i) OEA reduces food intake after systemic administration, but not after infusion into the brain ventricles (Rodríguez de Fonseca et al. 2001); (ii) local elevations in

small-intestinal OEA production mimic the hypophagic effects of exogenous OEA (Fu et al. 2008); (iii) surgical disconnection of the vagus nerve prevents such effects, but not those of centrally acting anorexiants (Rodríguez de Fonseca et al. 2001); (iv) a similar failure to respond to OEA is seen in rats treated with neurotoxic doses of capsaicin, which deprive the animals of peripheral vagal and non-vagal sensory fibers (Rodríguez de Fonseca et al. 2001); and, finally (v) IP injections of OEA stimulate transcription of c-Fos (a marker of neuronal activation) in the nucleus of the solitary tract (NST) (Rodríguez de Fonseca et al. 2001, Gaetani et al. 2010) (Figure 7).

From the brainstem nucleus of NST, the signal is relayed to magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, stimulating oxytocin neurotransmission (Gaetani et al. 2010). 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). This neural peptide, whose ability to control satiety is most likely mediated by activation of descending projections from hypothalamus to brainstem, plays an obligatory role in OEA-induced satiety. 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 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). Using two distinct experimental paradigms in rats (inhibitory avoidance and the Morris watermaze) Campolongo et al. found that i.p. administration of OEA after behavioural training strongly improved the retention of these tasks. This effect was blocked by infusions of the local anaesthetic lidocaine into the NST or the β-adrenergic antagonist propranolol into the BLA, indicating that the signal launched by peripheral OEA gains access to the CNS via the afferent vagus, and strengthens memory consolidation by stimulating noradrenergic activity in the BLA (Campolongo et al. 2009) (Figure 7). 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.

A recent study conducted by Misto et al (Misto et al. 2019) demonstrated that mast cells contribute to the control of fasting-induced ketogenesis through a paracrine mechanism, which involves secretion of histamine into the hepatic portal circulation, stimulation of liver H_1 receptors, and local biosynthesis of OEA.

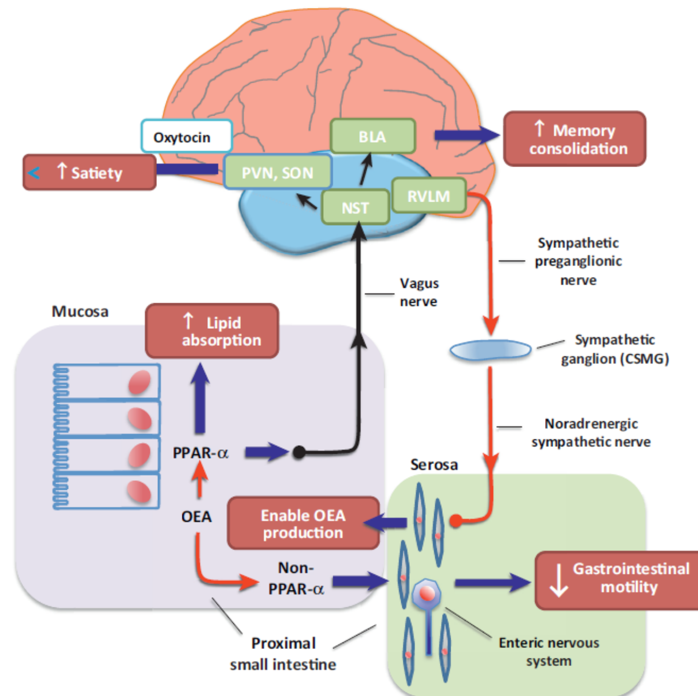


Figure 5. From gut to brain and back. The entry of oleic acid into gut mucosal cells stimulates OEA mobilization, which launches a PPAR-α-mediated signal that travels to the CNS through the afferent vagus nerve. From the the solitary tract (NST), the signal is relayed to magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, stimulating oxytocin neurotransmission. The PPAR-a-mediated signal may also travel to the basolateral nucleus of the amygdala (BLA) to strengthen the consolidation of recently formed memories. Within the gut, OEA may influence fatty acid (FA) absorption (through PPAR-α) as well as smooth-muscle motility (through an as-yet unidentified receptor). A descending sympathetic pathway, presumably originating in the rostral ventrolateral medulla (RVLM), facilitates OEA production by activating b-adrenergic receptors in the submucosal layer and the myenteric plexus (Piomelli 2013).

Other behavioural effects of OEA include antidyskinetic effect as assessed by using a hemiparkinsonian model of Parkinson's diseases (PD) in mice by using 6-OHDA striatal lesion. OEA treatment reduced axial, forelimb and orolingual dyskinetic symptoms, as well as contralateral rotations induced by 6-OHDA (González-Aparicio and Moratalla 2014). Also, OEA reduced spontaneous locomotor activity and attenuated psychomotor activation induced by cocaine, an effect that does not seem to be mediated by PPARα (Bilbao et al. 2013). OEA's central effects were also tested in depressive-like behaviour by using two animal models: the chronic unpredictable mild stress (CUMS) and tail suspension/forced swim tests. OEA treatment normalized sucrose preferences, rearing frequencies, prefrontal cortex and

hippocampal atrophy and reversed the abnormalities of BDNF and MDA levels and SOD activities in the hippocampus and prefrontal cortex, as well as changes in serum levels of ACTH, CORT, and T-AOC in CUMS, demonstrating antioxidant properties and normalisation of the hyperactivity in the HPA (Jin et al. 2015) .In the tail suspension test (TST) and/or forced swimming test (FST), OEA treatment decreased the immobility time demonstrating an antidepressant-like effect and also and increased cerebral levels of NE and 5-HT regulating central monoamine neurotransmitters (Yu et al. 2015a).

OEA recruits numerous neurotransmitters and food-regulating hormones to control feeding behaviour among which histamine, which regulates the homeostasis and essential functions in the brain, including eating pattern and circadian rhythms (Romano et al. 2015).

A study of our laboratory demonstrated that disruption of histidine decarboxylase (HDC), the primary enzyme for regulating histamine biosynthesis, lowers the hypophagic actions of OEA (Provensi et al. 2014). Brain histamine affects feeding behaviour and it is fundamental for appetitive and aversive responses during motivated behaviour, and blockade of histamine H_1R in the hypothalamus is believed to be responsible for the weight gain and metabolic dysregulation associated with the clinical use of atypical antipsychotics (Kim et al. 2007). Provensi and co-workers (2014) demonstrated that lack of central histamine dampens OEA-induced increase of c-Fos expression in oxytocin PVN neurons. Therefore, OEA requires the integrity of the brain histaminergic system to fully exert its hypophagic effect. These findings establish new functional connections between peripherally acting hypophagic signals and brain histamine neurotransmission (Provensi et al. 2014).

Provensi et al. showed that OEA increases memory expression of an aversively motivated task, contextual fear conditioning, by eliciting histaminergic neurotransmission in the BLA. Accordingly, both depletion of releasable histamine in the brain with α-fluoromethylhistidine (α-FMH) that blocks the histamine synthesizing enzyme histidine decarboxylase, and intra-BLA infusion of histaminergic antagonists prevent the freezing-enhancing effects of OEA (Provensi et al. 2017).

As previously reported, OEA has a potential antidepressant effect since it reduces immobility time in the TST (Jiang et al. 2015, Yu et al. 2015a). Our laboratory recently demonstrated that the reduction of immobility time occurred in normal, but not in histamine-deprived mice and, as other antidepressant compounds, OEA elicited CREB phosphorylation, one of the molecular mechanisms associated to the efficacy of SSRI treatment (Carlezon, Duman and Nestler 2005) in normal mice, but not in brain histamine-deprived mice, nor in PPAR-$\alpha^{-/-}$ mice, suggesting

that these nuclear receptors play an important role in the antidepressant-like properties of OEA (Costa et al. 2018).

Can brain histamine modulate social-stress induced effects?

Anatomic Framework

In the central nervous system, the presence of histamine is attributed to the presence of histamine-releasing neurons. The amount of histamine derived by non-neuronal pool (mast cells) is somewhat limited under normal conditions. Other possible sources of histamine in the brain may include microglia and microvascular endothelial cells (Katoh et al. 2001, Yamakami et al. 2000). Mast cells are relatively scarce in the brain, in comparison to other tissues, and their function is at present unclear. Furthermore, peripherally synthetized histamine does not contribute to its central content due to the histamine inability to cross the blood brain barrier. Therefore, it can be assumed that central histaminergic function are due almost exclusively to histaminergic neurons (Brown and Ennis 2001). The histamine-producing neurons are located in the small tuberomamillary nucleus (TMN). The name derives from the anatomical term *tuber cinerum*, denoting an ashen swelling located rostral to the mammillary bodies and caudal to the optic chiasm, forming the floor of the third ventricle in the hypothalamus (Krüger and Nyland 1995). The TMN in rats has been subdivided by Ericson *et al.* (Ericson, Watanabe and Köhler 1987) into three subgroups: (I) the medial tuberomamillary subgroup (TMM), which consists of about 600 neurons located on either side of the mamillary recess; (II) the ventral tuberomamillary subgroup (TMV), which contains approximately 1500 neurons around the mamillary bodies; and (III) the diffuse part of the TM (TMdiff or E5), which is made up of about 100 HD-immunoreactive perikarya scattered within or between various hypothalamic nuclei (Inagaki et al. 1990). In the mouse brain the TMN is less compact and is characterized by smaller and fewer neurons than rat TMN (Parmentier et al. 2002). The human TMN consist of about 64.000 neurons anatomically identified as the ventral, medial area and the lateral area (Airaksinen et al. 1991).

The histamine neurons in the TMN send projections that innervate the entire brain, and parts of the spinal cord (Figure 8) (Panula, Yang and Costa 1984, Watanabe et al. 1984). Two ascending pathways and one descending pathway have been identified (Panula et al. 1989). The highest density of histaminergic fibres are found in the hypothalamus, diagonal band, septum and olfactory tubercle. Moderate density of fibres is found in cerebral cortex, striatum and nucleus accumbens. Projections to the midbrain, brain stem, cerebellum and spinal cord tend to be of

lower density. The hippocampal formation is most strongly innervated in the subiculum and dentate gyrus, with a low density of fibres present in CA3 and CA1(Brown and Ennis 2001). The afferent projections to TM neurons are widespread and come from many different areas. Prominent sources are the infralimbic prefrontal cortex, lateral septum and preoptic nucleus (Ericson, Blomqvist and Köhler 1991). Most of the efferent histaminergic fibres are unmyelinated and except for those that project to the trigeminal nucleus (Inagaki et al. 1988), do not in general form synaptic specializations, rather, histamine is released from varicosities located periodically along the axon (Takagi et al. 1986). Thus, histamine release sites and histamine receptors are not directly associated to one another. Rather, histamine has been proposed to act like a local hormone on neurons, glial cells and blood vessels in a concerted manner (Wada et al. 1991). In addition to histamine, the TMN neurons contain several other neurotransmitters and modulators like GABA that is presumably released in specific brain regions to modulate behavioural response (Williams et al. 2014, Yu et al. 2015b). The neuropeptides Galanin, thyrotropin-releasing hormone, proenkephalin-derived peptides and substance P are also found in various populations of histamine producing TM neurons.

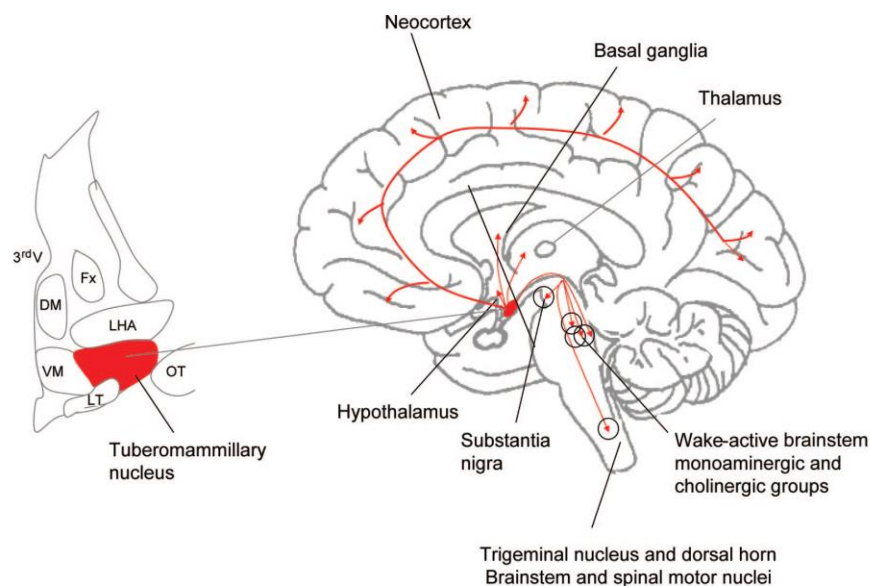


Figure 8. The brain histaminergic system. The tuberomammillary nucleus (TMN) of the hypothalamus is the sole source of histaminergic innervation of the CNS. Varicose axons of TMN neurons provide widespread input to all areas of the CNS via 2 ascending pathways that innervate the hypothalamus, basal forebrain, basal ganglia, amygdala, hippocampus, and cerebral cortex and 1 descending pathway that innervates the brainstem, including the cholinergic and monoaminergic nuclei, cerebellum, and spinal cord. (Benarroch 2010)

Histaminergic Receptors

The basic homeostatic and higher functions, including cognition, arousal, circadian and feeding rhythms regulated by brain histamine are due to the action on 4 metabotropic receptors: H_1R, H_2R, H_3R, H_4R. All of histaminergic receptors are expressed at central level with different density in different brain regions (Passani and Blandina 2011). All metabotropic histamine receptors (H_1R-H_4R) belong to the rhodopsin-like family of G protein coupled receptors (GPCR). Each receptor consists of seven large transmembrane-spanning elements with prototypic domains (Haas et al. 2008). Three of the four histamine receptors that have been identified (H_1–H_3) are prominently expressed in the brain in specific cellular compartments, whereas the fourth (H_4) receptor is detected predominantly in bone marrow and leukocytes (Haas and Panula 2003).

Histamine H_1 Receptor

The human H_1 receptor is encoded by a gene of 56kDa composed by 487~490 amino acids located on chromosome 3p25 (Jongejan et al. 2005). The signal transduction of H_1R is mainly mediated by coupling to Gq/11 proteins (Gutowski et al. 1991, Leopoldt, Harteneck and Nürnberg 1997, Selbach, Brown and Haas 1997, Moniri, Covington-Strachan and Booth 2004), but also signals via Gi/o in some systems (Seifert et al. 1994, Wang and Kotlikoff 2000), and the small G protein family, most likely through an indirect downstream effect (Mitchell and Mayeenuddin 1998). The interaction of H_1 receptor with Gq/11 protein and phospholipase C promotes inositol trisphosphate (IP3)-dependent Ca2+ release from intracellular Ca^{2+}-stores, and also diacylglycerol formation. H_1R also activates AMP-kinase, nuclear factor kappa B, nitric oxide synthases, and phospholipase A2 (PLA2), which induces arachidonic acid formation (Haas et al. 2008). H_1R are found throughout the whole body and nervous system. H_1 receptors are widely distributed in mammalian brain (Hill 1990, Schwartz et al. 1991). High densities are found in brain regions concerned with neuroendocrine, behavioural, and nutritional state control, including the periventricular, suprachiasmatic, and ventromedial nuclei of the hypothalamus, aminergic and cholinergic brainstem nuclei, thalamus, and cortex (Schwartz et al. 1991). The global loss of H_1R in KO mice produces immunological, metabolic, and behavioural abnormalities (Masaki and Yoshimatsu 2006, Hirai et al. 2004, Huang et al. 2006, Haas et al. 2008).

Histamine H_2 Receptor

A second class of histamine receptors was identified by Black and colleagues based on the different pharmacological profile of the histamine receptor responsible for stimulating gastric acid secretion (Hill et al. 1997). The gene encoding the human H_2R, which is a 40-kDa 359-amino acid peptide, is located on chromosome 5q35.5. H_2R couple to Gs proteins to stimulate adenylyl cyclase and increase intracellular cAMP, which activates protein kinase A (PKA) and the transcription factor CREB, all of which are key regulators of neuronal physiology and plasticity. Through H_2R activation and PKA-dependent phosphorylation, histamine blocks a Ca^{2+}-activated potassium conductance responsible for the neuronal excitability (Haas et al. 2008). Independent of either cAMP or [Ca^{2+}]i levels, H_2R also inhibit PLA2 and release of arachidonic acid, which likely account for the opposing physiological responses elicited by H_1R and H_2R in many tissues (Traiffort et al. 1992). Like the histamine H_1 receptor, the H_2 receptor has a widespread expression in the brain and spinal cord, particularly high densities are found in the basal ganglia and in parts of the limbic system such as the hippocampal formation and amygdala. In contrast to H_1 receptors, H_2 receptors are present in low densities in septal areas, hypothalamic and thalamic nuclei. H_1 and H_2 receptors are colocalized in several areas of the brain including pyramidal and granule cells in the hippocampal formation and in the other aminergic cell where the receptors can act synergistically, e.g. in the stimulation of cAMP production (Brown and Ennis 2001). Mice deficient in H_2R function exhibit selective cognitive deficits along with an impairment in hippocampal LTP (Dai et al. 2007) and with abnormalities in nociception (Mobarakeh et al. 2005, Mobarakeh et al. 2006) and gastric and immune functions (Teuscher et al. 2004).

Histamine H_3 Receptor

Histamine H_3 receptor in the brain were detected in1983 by the group of J.C. Schwartz in Paris proved its neurotransmitter function as auto- as well as hetero-receptor at pre- and postsynaptic membranes and revealed its profound influence on different neurotransmitter balances (Panula et al. 2015). The gene (Hrh_3) encoding human H_3R, a 70-kDa 445-amino acid peptide, is located on chromosome 20q13.33. H_3R negatively couple through pertussis toxin-sensitive Gi/o proteins to N- and P-type Ca^{2+} channels and to adenylyl cyclase. Through extensive cross-talks with other GPCRs, they can also engage Gq/11 signalling and activate PLA2, AKT/GSK3, and MAP kinase pathways, all of which play important roles in axonal and synaptic plasticity and a variety of brain disorders (Haas et al. 2008). The histamine H_3 receptor is located on histaminergic neuron somata, dendrites and axon varicosities, as well as on the axon varicosities

and somata of other neurons, providing negative feedback to inhibit histamine synthesis and the release of histamine or other transmitters, including glutamate (Brown and Reymann 1996, Doreulee et al. 2001), acetylcholine (Arrang, Morisset and Gbahou 2007, Passani and Blandina 1998) and GABA (Jang et al. 2001, Yamamoto et al. 1997). In keeping with their role as auto- and hetero-receptors, H_3R are heterogeneously distributed among areas known to receive histaminergic projections (Hu and Chen 2017). The CNS contains the great majority of H_3Rs, although they can also be found in the periphery such as heart, lung and intestine (Hancock et al. 2003). In rodents, high H_3Rs densities are found particularly in anterior parts of the cerebral cortex, hippocampus, amygdala, nucleus accumbens, striatum, olfactory tubercles, cerebellum, substantia nigra, and brain stem. In the TMN, H_3R reside on perikarya of histaminergic neurons. Loss of H_3R function in KO mice is associated with behavioural state abnormalities, reduced locomotion (Toyota et al. 2002), a metabolic syndrome with hyperphagia, late-onset obesity, increased insulin and leptin levels (Tokita, Takahashi and Kotani 2006, Yoshimoto et al. 2006), and an increased severity of neuroinflammatory diseases (Teuscher et al. 2007).

An important feature of the H_3R is its high degree of constitutive or spontaneous activity (Morisset et al. 2000). As a G-protein-coupled receptor, the H_3R is an allosteric protein that can adopt various conformations in equilibrium and the spontaneously active state leads to constitutive activity. It has been suggested that the potency of various H_3R antagonist might depend on their intrinsic activity as inverse agonists. This discovery is important for drug development because the ability to compete with constitutively active H_3R states might have important therapeutic implication. Currently, an H3R antagonist/inverse agonist, Pitolisant, in 2016 was introduced for clinical use for the treatment of narcolepsy (Syed 2016). Moreover, other H_3R antagonist/inverse agonist are in phase II and phase III clinical trial for potential treatment of cognitive impairment associated with Alzheimer’s disease and Parkinson’s disease(Kubo et al. 2015), schizophrenia, attention deficit hyperactivity disorders and obesity (Passani and Blandina 2011).

Histamine H_4 Receptor

Six independent laboratories contributed to the identification and cloning of the H_4 receptor (Nakamura et al. 2000, Oda et al. 2000, Liu et al. 2001, Morse et al. 2001, Nguyen et al. 2001, Zhu et al. 2001, O'Reilly et al. 2002). The human H_4 receptor gene is present on chromosome 18q11.2 and is a 44 kDa 390-amino-acid polypeptide. Like H_3R, the H_4 receptor is coupled to pertussis toxin sensitive Gi/o protein with inhibitory effect on cAMP accumulation (Oda et al. 2000, Leurs et al. 2009). H_4 receptor expression has been observed in eosinophils, T cells,

dendritic cells, basophils, and mast cells (Liu et al. 2001, Gantner et al. 2002, O'Reilly et al. 2002, Hofstra et al. 2003), but its expression in the central nervous system remains controversial. In the human brain, expression of H_4 receptor mRNA has been reported in the amygdala, cerebellum, corpus callosum, cortex frontal cortex, hippocampus, and thalamus (Strakhova et al. 2009) but, results obtained with analyses of mRNA expression does not always reflect results obtained with immunohistochemistry, therefore there is a debate about H_4R in SNC that needs further research (Panula et al. 2015).

Homeostatic Histaminergic Functions

The morphology of brain histaminergic system with a compact group of cells and a capillary distribution of varicose fibres suggests its action as a normative centre for brain activity. Pharmacological studies in intact and histamine-deficient animals as well as humans link brain histamine with homoeostatic brain functions and neuroendocrine control. Brain histamine controls behavioural responses, biological rhythms, body weight, energy metabolism, thermoregulation, fluid balance, stress, and reproduction (Hough 1988, Schwartz et al. 1991, Parmentier et al. 2002).

Our laboratory demonstrated functional differences in TMN neurons, suggesting that histaminergic neurons are organized in distinct subpopulation impinging on different brain regions (Giannoni et al. 2009, Blandina et al. 2012).

Sleep and Wakefulness

Histaminergic neurons help sustain wakefulness. Several studies corroborate this hypothesis; in H_1R-KO mice the sleep-wake cycle is impaired, and the waking promotion induced by H_3R antagonist is abolished (Huang et al. 2006, Lin et al. 2002). During waking c-fos expression increases in TMN neurons (Lin 2000, Nelson et al. 2002, Nelson et al. 2003, Scammell et al. 2000, Sherin et al. 1998, Vanni-Mercier et al. 2003). The regulation of the transition between wakefulness and sleep involves antagonist influences of sleep-promoting VLPO neurons, which provide inhibitory GABA- and galanin-mediated inputs to TMN and brainstem cholinergic and monoaminergic groups, and excitatory effects of orexin (Hcrt/Orx) neurons on TMN and other wake-active neuronal groups (Benarroch 2010). TMN neurons become active just after waking and fire at an average rate of about 5 Hz, and their activity is suppressed during sleep (Sakai et al. 2010, Saper et al. 2010, Takahashi, Lin and Sakai 2006). A recent elegant work by Wisden and co-workers demonstrated that zolpidem, a $GABA_A$ receptor-positive modulator, needs to work on specific cell types of the brain, including histaminergic neurons, to induce sleep,

without reducing the power of the sleep, hence improving sleep quality (Uygun et al. 2016). Furthermore, the same laboratory showed that wake-active histaminergic neurons generate a paracrine GABAergic signal that serves to provide a brake on over-activation from histamine, but could also increase the precision of neocortical processing (Yu et al. 2015b).

Feeding and Energy Metabolism

The evidence for histamine involvement in food intake is nowadays very consistent (Provensi, Blandina and Passani 2016a, Provensi et al. 2014). Early studies demonstrated that i.c.v injection of histamine, loading with histamine precursor L-histidine or application of the H_3 receptor antagonist thioperamide suppress feeding (Cohn, Ball and Hirsch 1973, Sheiner, Morris and Anderson 1985, Machidori et al. 1992, Ookuma et al. 1993), whereas i.c.v infusions of α-FMH or H_1 receptors antagonists increase food intake (Sakata et al. 1988b, Fukagawa et al. 1989, Ookuma et al. 1989, Sakata et al. 1988a). However, the role of histamine is not restricted to feeding control but also the regulation of body weight and adiposity by modulation of peripheral energy. Many of the central hypothalamic areas involved in regulating feeding, including the arcuate, ventromedial (VMH) and paraventricular (PVN) nucleus and lateral hypothalamic perifornical area (LHA), are densely innervated by histamine containing fibres and show a high density of H_1Rs (Panula et al. 1989). Early work suggested that histamine-mediated suppression of food intake was controlled by the VMH as microinfusion of H_1R antagonists into the VMH but not PVN or LH elicited feeding responses and increases both meal size and duration (Fukagawa et al. 1989, Sakata et al. 2003). Likewise, electrophoretic application of H_1R antagonists suppressed the firing of glucose-responsive units in the VMH but not in the LHA or PVN (Fukagawa et al. 1989). Another site of importance in the histamine control of food intake is the mesencephalic trigeminal nucleus. Bilateral injections of α-FMH into this region reduced eating speed and prolonged meal duration while leaving meal size unaltered. Feeding induced increases in histamine turnover in both the trigeminal nucleus, which controls mastication, and the ventromedial area, which is considered as a satiety centre (Fujise et al. 1998). In our laboratory, we recently showed that the PVN as well takes part into the histaminergic control feeding behaviour as histamine released in the PVN activates oxytocin neurons (Provensi et al. 2014) that in turn exert hypophagic behaviour (Gaetani et al. 2010). Furthermore, the orexigenic actions of orexins/hypocretins (Jørgensen et al. 2005) and the anorexigenic effects of leptin (Toftegaard et al. 2003) and glucagon-like peptide-1 (GLP-1), which depend on CRH released by PVN neurons (Gotoh et al. 2005), are all blunted or absent by pharmacological or genetic loss of H_1R function. Ghrelin, another peptide of peripheral

origin, does not affect histamine release, suggesting that ghrelin may act on a parallel, different mechanism that controls food intake (Ishizuka et al. 2006).
Most experimental observations in rodents agree that blockade of brain H_3 receptor, hence increasing histamine release, decreases energy intake, body weight and plasma triglycerides (Hancock and Brune 2005). Also, they increase histamine release from the hypothalamus, they reduce energy intake in normal and leptin-resistant mice with diet induced obesity (Ishizuka et al. 2008), and decrease food intake in wild type mice (Provensi et al. 2014).

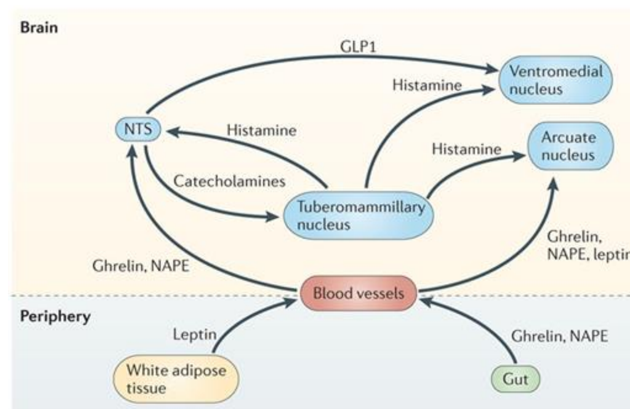


Figure 9. Key brain areas involved in the regulation of feeding and their innervation by histaminergic fibres (Panula and Nuutinen 2013).

From the pharmacological point of view the importance of histamine in the regulation of feeding behaviour came from the observation that increased weight is a common adverse effect of many classic antipsychotic drugs and atypical antipsychotics that depends on their affinity as antagonists at the H_1Rs (Kroeze et al. 2003). Preclinical studies showed that activation of histamine neurons induces the arousal state during food anticipation (Inzunza et al. 2000, Angeles-Castellanos, Aguilar-Roblero and Escobar 2004), and during the appetitive phase that precede food consumption (Passani and Blandina 2011).

Cognitive functions of brain histamine

Eric Kandel defined learning and memory as a continuous process, and he stated that, "Learning is the process by which we acquire knowledge about the world and memory is the process by which that knowledge of the world is encoded, stored, and later retrieved" (Kandel et al. 2012). Learning is the process that modifies subsequent behaviour while memory is the ability to remember past experiences. Cognition, on the other hand, is a broad term that applies to processes such as memory, association, language, attention, concept formation and problem solving (Coren, Porac and Ward 1984).

Memory can be divided into short-term (working memory) and long-term memory. Short-term memory has a limited capacity and lasts only for a period of several seconds to a minute. In contrast, long-term memory can store larger quantities of information for potentially unlimited duration. Long-term memory is divided into declarative (explicit) or non-declarative (implicit) memory. Declarative memory answers the question “what”, and it includes knowledge of facts such as places, things and people, and the meaning of these facts. Declarative memory is further sub-divided into episodic memory, which is the personally experienced event specific to a particular context such as time and place; and semantic memory, which involves knowledge of these facts taken independent of the context in which they were learned (Miller 1956, Tulving 1972).The major brain structure involved in declarative memory is the hippocampus along with other medial temporal lobe structures (Squire and Zola 1996). Non-declarative or implicit memory, on the other hand, answers the question “how”. It is the acquisition of motor skills and habits and is mediated by neostriatum and cerebellum (Bechara et al. 1995, Knowlton, Mangels and Squire 1996, Salmon and Butters 1995). In addition, the amygdala mediates emotional memory and has been shown to be involved in memory consolidation (Cahill et al. 1995).

Fear memory

Forming associations about events and then consolidating memories of those associations is an important strategy for survival. However, in traumatic situations, these associations sometimes become overly consolidated and then, potentially, are resistant to extinction over time, resulting in fear-related disorders (Parsons and Ressler 2013). But fear, in general, has a strong survival value. The lack of fear, also called recklessness or mindlessness in humans, is inherently dangerous and potentially lethal (Izquierdo, Furini and Myskiw 2016). Therefore, in both cases, over-consolidation/resistant-extinction and recklessness/loss of fear are two side of the same coin, dangerous in the same way.

The acquisition and memory of conditioned fear depend on both hippocampus and amygdala, as lesion studies (Lorenzini et al. 1996b, Lorenzini et al. 1996a, Sacchetti et al. 1999, Sacchetti et al. 2002) and biochemical studies (Trifilieff et al. 2007) indicate. The sensory-related information from hippocampus and amygdala presumably originates in the mesencephalic reticular formation and ventral tegmental area (VTA), which receive it, in turn, from collaterals of the sensory pathways (BUSER and ROUGEUL 1961, GREEN and MACHNE 1955, MACHNE and SEGUNDO 1956). The hippocampus and the basolateral amygdala (BLA) together with the ventro-medial prefrontal cortex (vmPFC) orchestrate memory formation

(Izquierdo et al. 2016). Also, the periaqueductal grey (PAG), is a brain region that conveys aversive signals to the amygdala. The PAG is known as an output structure for various conditioned fear responses, it receives a strong nociceptive input from the spinal and trigeminal dorsal horn (Gross and Canteras 2012) and a recent study found that temporary pharmacological inactivation of PAG reduces shock-evoked responding in amygdala neurons and the acquisition of fear learning (Johansen et al. 2011).

How to evaluate fear memory in rodents: most widely used paradigms

Fear learning is usually studied by classical (Pavlovian) or instrumental association between the environment or changes in the environment (conditioned stimulus, CS) and a fearsome stimulus (usually one or more mild foot shocks; unconditioned stimulus, US). This type of learning represents situations in humans in which initially neutral stimuli become threatening through pairing with other stimuli and generate fear, a human emotion that guides much of our behaviour and is crucial for survival (Izquierdo et al. 2016).

In the fear conditioning paradigm, the animals are placed in a new environment (context) were they receive a mild aversive stimulus such as a foot shock (Unconditioned Stimulus, US) associated with another stimulus such as tone or light (Conditioned Stimulus, CS), that usually does not elicit a response. Following learning, the presentation of the CS alone generates various visceral and behavioural conditioned fear responses. The term fear response is used to refer specifically to measurable responses that occur in response to threat and not to the conscious feelings of fear: called freezing behaviour. Freezing behaviour (conditioned response, CR), is a generalized immobility caused by a generalized tonic response of the animals' skeletal musculature except those muscles used in breathing (Herry and Johansen 2014, Izquierdo et al. 2016).

Promnesic agents are expected to increase, whereas amnesic manipulations to reduce freezing behaviour (Wehner and Radcliffe 2004, Curzon , Rustay and Browman 2009).

The most widely used instrumental fear conditioning is one-trial inhibitory avoidance (IA) (Gold 1986, Izquierdo et al. 2006, Izquierdo and Medina 1997) which used to be called “passive avoidance” in opposition to the “active avoidance” tasks in which animals have to perform some movement to avoid the foot shocks. In the “passive” tasks, animals have to withhold stepping through a hole into a dark compartment, or stepping down from a platform onto a grid, to access the shock compartment; the required response is to remain in the safe, lit compartment or on the start platform. Animals learn to avoid stepping through or stepping down, but they are not in any way refrained from moving or behave in any way passively. In fact, they move a lot

while on the platform or in the lit compartment (Netto and Izquierdo 1985). When re-tested, an increase in the latency to step-through or to step-down is related as a measure of learning (Izquierdo and McGaugh 2000).

Multiple systems and brain areas are involved in processing this kind of memories; the amygdala has been proven to be a key region engaged in mediating emotional valence during memory consolidation (Gold et al. 1975, Izquierdo and Medina 1997, McGaugh 2013, LeDoux 2003). The amygdala also modulates memory consolidation through projections to other brain regions, the cortex (Packard and Goodman 2012) and the hippocampus (Packard, Cahill and McGaugh 1994, McIntyre et al. 2005).

Does histamine have a role in aversive memory?

Several studies suggest that histaminergic neurons detect stress-induced signals and coordinate their influences on memory consolidation. First of all, emotionally arousing events activate the neuronal histaminergic system (Passani and Blandina 2011, Torrealba et al. 2012). Studies demonstrate that histamine regulates the consolidation of emotional memories. Almeida and Izquierdo reported more than 30 years ago that an i.c.v. injection of histamine immediately post-training ameliorated the performance of rats in the retention test of a step-down inhibitory avoidance paradigm measured 24 h later. This effect involved the activation of both H_1 and H_2 receptors (de Almeida and Izquierdo 1986).

Consistently, systemic treatment with H_3 receptor antagonists, known to increase synaptic levels of endogenous histamine by blocking inhibitory histamine autoreceptors (Arrang et al. 2007), enhanced the performance of rat pups in a multi-trial, inhibitory avoidance response, a task modelling aspects of ADHD and other disorders in which vigilance, impulsivity and/or cognitive performance are impaired (Fox et al. 2002, Komater et al. 2003).

Consistent with a facilitatory effect of H_3 receptor blockade on memory, thioperamide, an H_3 antagonist, facilitated, while Imetit, an H_3 agonist, impaired retention in mice trained on foot shock avoidance in a T-maze (Flood, Uezu and Morley 1998).

Recognition memory

Recognition memory confers the ability to learn and memorize the novelty of entities (Rossato et al. 2007). Recognition memory involves at least two separable processes, familiarity discrimination and recollection and more complex aspects of contextual, associative and spatial aspects (Brown and Banks 2015). Discrimination for visual stimuli seems to be affected by a system centred on the perirhinal cortex of the temporal lobe (Brown and Banks 2015). Regions

other than perirhinal cortex may also be involved in recognition memory processes: when a recognition memory task is solved using recollection or association concerning a presented stimulus, the recognition memory predicts hippocampal involvement (Brown, Warburton and Aggleton 2010). The hippocampus is involved in recognition memory using multiple items and associative or spatial information (Aggleton and Brown 2006, Dere et al. 2006, Eichenbaum, Yonelinas and Ranganath 2007, Murray and Ranganath 2007, Squire, Wixted and Clark 2007, Winters, Saksida and Bussey 2008). In particular, lesions of the rat hippocampus impair recognition memory that requires spatial information; location tasks are impaired by hippocampal lesions while perirhinal lesions have no effect (Warburton and Brown 2010).
Functional imaging in human subjects has implicated the prefrontal cortex in recognition memory processes (O'Neil et al. 2012). The role of the rodent medial prefrontal cortex in recognition memory has been extensively studied. Large lesions of the prefrontal cortex, which included the anterior cingulate, prelimbic and infralimbic cortices, or which centred on the ventral medial prefrontal cortex, produced recognition impairments (Kolb et al. 1994, Ragozzino, Detrick and Kesner 2002). The medial prefrontal cortex has been implicated in attentional processing (Muir 1996, Chudasama and Robbins 2003), and play an important role in temporal order memory (Mitchell and Laiacona 1998, Hannesson et al. 2004, Chiba, Kesner and Gibson 1997). Lesions in the medial prefrontal cortex impaired temporal order memory task (Barker et al. 2007, Devito and Eichenbaum 2011) but not induces deficits in the recognition or location tasks (Barker et al. 2007). Moreover, in humans and non-human primates damage to the medial dorsal thalamus (MD nucleus) produces recognition memory deficits (Victor 1987, Parker, Eacott and Gaffan 1997, Warburton and Brown 2015).

How to evaluate recognition memory in rodents: most widely used paradigms

Although highly conserved among species, the expression of declarative memories varies greatly among different species (Paul, Magda and Abel 2009). Declarative memory in humans, for example, is formulated through language and other explicit representations. Animals, on the other hand, cannot represent such knowledge verbally or symbolically, and different tests have been used as models of episodic memory tasks in rodents (Fouquet, Tobin and Rondi-Reig 2010). The most used tasks are the object recognition task and its variations (Ennaceur and Delacour 1988, Leger et al. 2013). The novel object recognition and the novel spatial location tests rely on the motivational strength of novelty, as they are based on the natural tendency of rodents to search and explore novel objects or the new location where an object has been displaced. These procedures have become popular methods for studying emotionally neutral

memories as they do not require punishments, food or water restriction, and several behavioural endpoints can be rapidly obtained, including general activity, reactivity to novelty, and learning (Blaser and Heyser 2015). Experimental animals usually remember objects previously encountered in an open arena and their location and spend more time exploring new objects or their new location. Usually, this type of memory is labile and does not last for more than 6–12 h (da Silveira et al. 2013b).

Does histamine have a role in recognition memory?

As previously reported, manipulation of the central histaminergic system during different learning paradigms modifies animals' behaviour. Early work by the group of Blandina et al. (1996) described the effects of systemic administration of the H_3 receptor agonists Imetit and R-α-methylhistamine prior to the acquisition session in the object discrimination test. The memory of treated animals was impaired, as they showed no significant differences in the time spent exploring either object (Blandina et al. 1996). Furthermore, scopolamine-induced memory impairment was prevented by pretreatment with the H_3 antagonists thioperamide or Clobenpropit (Giovannini et al. 1999).

Nowadays, most experimental observations agree that H_3 receptor antagonists prolong recognition memory and prevent anterograde or retrograde, pharmacologically induced memory impairment. In some studies, memory facilitation occurred when the H_3 receptor antagonist ciproxifan was given before the retention session (Pascoli, Boer-Saccomani and Hermant 2009) or when administered shortly after training or before retention sessions (Trofimiuk and Braszko 2014). These results are in agreement with the majority of studies indicating that H_3 receptor blockade facilitates retrieval. As mentioned above, H_3 receptor antagonists increase histamine release as well as the release of other neurotransmitters in brain regions crucial for the maintenance of alertness and storage of information. In keeping with this hypothesis, we demonstrated that administration of the non-imidazole H_3 receptor antagonist, ABT-239, to wild-type mice before training and retention test improved memory in the object recognition paradigm; the efficacy of ABT-239 on memory was not observed in the brain of histamine-depleted mice, suggesting that endogenous histamine is crucial for the mnemonic effects of H_3 receptor ligands (Provensi et al. 2016b).

Many studies, however, have evaluated the roles of other specific histaminergic receptors such as H_1 or H_2. Intra-hippocampal infusion of the H_1 receptor antagonist pyrilamine 30–120 min after training impaired recognition memory, whereas no effects on retention were observed when the drug was infused immediately or 360 min after acquisition. Similar findings were

observed when the H_2 receptor antagonist ranitidine or the H_3 receptor agonist Imetit were directly delivered into the CA1 region of the hippocampus (da Silveira et al. 2013a).
The participation of the central histaminergic system in recognition memory was also studied using genetically modified animals. It was observed that H_1 receptor as well as H_2 receptor deficiency impairs learning and memory, including object recognition (Dai et al. 2007). Mice lacking the H_1 receptor display also episodic like memory impairments as evaluated in a complex spatial and temporal object recognition task (Dere et al. 2006). Whereas H_3 receptor-knockout mice performed better than wild-type mice in a spatial learning task but not in novel object recognition learning (Rizk et al. 2004). The results of this studies suggest that histamine facilitates learning and memory. Indeed, HDC-knockout mice show impairment in novel object location (Acevedo et al. 2006) and non-reinforced episodic object memory (Dere et al. 2003).
As discussed in one of the previous chapters, memory processes can be profoundly affected by stress. Trofimiuk and Braszko (2014) observed that daily restrained rats for 21 days were unable to differentiate the novel from the familiar objects when the retention session was performed 24 h after the acquisition. Acute ciproxifan treatment counteracted the deleterious effects of chronic restrain stress on long-term recognition memory (Trofimiuk and Braszko 2014). Moreover, researchers using the Flinders Sensitive Line (FSL), a suitable rat model to study emotional and cognitive deficits of depression-like symptoms (Eriksson et al. 2012, Gómez-Galán et al. 2013) show that the behavioural repertoire of FSL rats acutely treated with the H_3R antagonist Clobenpropit or saline was compared with that of Sprague–Dawley (SD) rats treated with saline. During the test session, performed 24 h after training, SD rats preferentially explored the novel objects, whereas saline-treated FSL rats showed no object preference. Treatment of FSL rats with Clobenpropit increased the recognition index to the same level observed in SD rats, indicating that the drug treatment restored recognition memory (Femenía et al. 2015).

Social memory

Social recognition is a recognition memory fundamental to form and consolidate social groups; hence, it is important for reproduction, species survival and the establishment of dominance hierarchies. In addition to these forms of long-term social recognition, rodents are also known to form transient, short-term memories of recently encountered individuals (Thor and Holloway 1982, Winslow and Insel 2004). In humans and other primates, individual recognition relies mostly on visual and auditory cues. Indeed, in the human brain, a specific visual association area, the right fusiform gyrus, appears to be critical for face recognition (de Waal 2000, Fisher

et al. 1998). In most other mammals, social information is encoded via olfactory or pheromonal signals, although auditory and visual signals may have important influences (Gowaty, Drickamer and Schmid-Holmes 2003).

Social memory performance depends on various factors, including the species, sex, age, and emotional status of the animal during the investigation. memory. A study (Noack et al. 2010) suggested that differences in olfaction between rats and mice might account for the species-specific differences in the social preference task. Thus, although the number of c-Fos-positive cells after exposure to the volatile scent of a juvenile was robustly upregulated in the accessory olfactory bulb (OB) and the main OB in mice, the same procedure in rats only modestly increased the number of c-Fos-positive cells and only in the accessory OB. Gender may also play a role in social recognition. A study showed that although the total investigation time of a conspecific juvenile was lower in female than in male Long-Evans rats, young female rats displayed stronger social recognition than males (Markham and Juraska 2007). In agreement with these findings, oestrogen replacement in ovariectomized mice was reported to be beneficial for social recognition (Tang et al. 2005). A more recent study identified the medial amygdala (MeA) as a site of action for the effects of oestrogen administration on social recognition (Spiteri et al. 2010). Interestingly, castration of male rats results in enhanced social recognition, supposedly by diminishing arginine vasopressin (AVP) function (Bluthé and Dantzer 1992). Of note, a role for AVP in social recognition has been established (Sekiguchi, Wolterink and van Ree 1991, Landgraf et al. 1998, Ferguson, Young and Insel 2002, Bielsky and Young 2004). Aging is generally accompanied by the deterioration of social recognition in both rats and mice (Terranova et al. 1994). For example, old (24-month-old) Wistar rats typically demonstrate reduced social recognition in juvenile compared to young (3-month-old) rats (Prediger, De-Mello and Takahashi 2006). Some studies have investigated the impact of the circadian cycle on social memory. Intracerebroventricular (ICV) infusion of melatonin or histamine—both of which are important modulators of the circadian rhythm—in rats facilitated social recognition (Prast, Argyriou and Philippu 1996, Argyriou, Prast and Philippu 1998). However, the relevance of this variable is unclear because disruption of the circadian rhythm was not found to affect social memory in rats (Reijmers et al. 2001).

Studies have shown that multiple brain regions regulate social behaviours and the formation of social recognition memory. The hippocampus, amygdala, and anterior cingulate cortex (ACC) are critical regions for the formation/consolidation of social recognition memory in mice (Kogan, Frankland and Silva 2000, Suzuki et al. 2011, Hitti and Siegelbaum 2014, Garrido Zinn et al. 2016). Conversely, the medial prefrontal cortex (mPFC) and amygdala are involved in the

regulation of social behaviours such as social interaction and approach (Jodo et al. 2010, Yizhar et al. 2011, Felix-Ortiz et al. 2016).
The study of the neurochemistry of social memories has largely concentrated on the 'social neuropeptides', oxytocin (OT) and vasopressin (AVP). OT and AVP are closely related neuropeptides that are produced in the paraventricular nucleus and supraoptic nucleus of the hypothalamus as well as in extra-hypothalamic sites (Castel and Morris 1988). They are currently acknowledged for their major involvement in social behaviour in both rodents (Bielsky and Young 2004, Ross and Young 2009) and humans (Meyer-Lindenberg 2008).
Brattleboro rats, for example, lack AVP production in the hypothalamus and do not recognize a juvenile conspecific encountered 30 min earlier. In turn, administration of AVP into the lateral septum restores social recognition in the Brattleboro rat without affecting social exploration per se (Engelmann and Landgraf 1994, Feifel et al. 2009). Moreover, animals lacking an AVP receptor AVP1bR show reduced interest in a conspecific when given the choice to explore a juvenile vs. an empty compartment but they did not show a significant social preference during the test, indicating a lack of social interest (DeVito et al. 2009). This suggests that AVP is important for the social component of social recognition because perturbations of this system do not appear to affect object recognition (DeVito et al. 2009).
As said before, OT also plays a very important role in social memory (Bielsky and Young 2004). ICV injections of OT facilitated social recognition of a juvenile in rats that could be blocked with an OT antagonist (Benelli et al. 1995, Dluzen et al. 1998, Samuelsen and Meredith 2011). OT KO mice from both genders showed impaired social recognition without alterations in social approach, olfactory functions or cognitive impairments (Ferguson et al. 2000, Crawley et al. 2007, Choleris et al. 2003). The social recognition deficit in OT KO mice was rescued through OT infusion (Ferguson et al. 2000, Ferguson et al. 2001). OT receptor (OTR) KO mice showed impaired social recognition (Takayanagi et al. 2005), in line with the findings obtained in OT KO mice.

How to evaluate social memory in rodents: most widely used paradigms

Social recognition is assessed in laboratory rodents using tests that represent different variants of a basic scheme that measures familiarity recognition. The basic principle relies on the usual propensity of rodents to investigate an unfamiliar conspecific more thoroughly than a familiar one. (van der Kooij and Sandi 2012).
The most widely used variant is the habituation/dishabituation paradigm (Thor and Holloway 1982). In a first step (habituation), a stimulus rodent is presented either once (Thor 1980, Thor

and Holloway 1982) or repeatedly (Choleris et al. 2003) to an experimental rodent that shows a reduction in its social investigative response on subsequent presentations. Once social investigation has declined, in a second step (dishabituation) the presentation of a different conspecific reinstates the initial level of social investigation. In this paradigm social recognition is inferred from a change in the behaviour of the experimental animal upon subsequent tests. This constitutes a potential disadvantage to the use of this procedure because repeated testing of the same animal can lead to non-specific behavioural changes, such as sensitization to the testing procedure (Engelmann and Landgraf 1994). These effects may mask specific social recognition related behavioural changes.

The social discrimination paradigm has proven to be more sensitive for measuring social recognition than the habituation/dishabituation test (Choleris et al. 2009) because is provided with a simultaneous binary choice between a novel and a familiar conspecific (Engelmann and Landgraf 1994, Choleris et al. 2006). The social discrimination paradigm shares the initial exposure to an unfamiliar conspecific with the habituation/dishabituation paradigm but differs in the subsequent phase. In this case, both a familiar and a novel conspecific are simultaneously presented. In some cases, the two stimulus animals are left to freely move about the cage, whereas in other studies, they are confined within wired cups which is frequently the case when a three-chambered apparatus is used (Silverman et al. 2010). The experimental design of this test allows evaluation of two critical but distinguishable aspects of social behaviour, such as social affiliation/motivation, as well as social memory and novelty. "Sociability" in this case is defined as the propensity to spend time with another mouse, as compared to the time spent alone exploring the empty cup (Moy et al. 2004). "Preference for social novelty" is defined as propensity to spend time with a previously unencountered mouse rather than with a familiar mouse (Moy et al. 2004).

Even though it is less popular and less utilized than the habituation/dishabituation procedure, the social discrimination paradigm has been shown to be more a sensitive means of assessing social recognition because it has allowed for the emergence of social discrimination in animals that appeared to possess no social recognition when tested in the habituation/dishabituation paradigm (Choleris et al. 2006, Engelmann and Landgraf 1994). For both paradigms various versions exist using different exposure times, inter-trial intervals and stimulus animals.

Does histamine have a role in social memory?

Neurotransmitters such as noradrenaline, dopamine and acetylcholine (Griffin and Taylor 1995, Di Cara et al. 2007, Deiana, Platt and Riedel 2011) and hormones such as oxytocin (Raam et

al. 2017, Lin et al. 2018) have been suggested to play key roles in social discrimination and memory. Early work by Philippu and colleagues showed that histamine is also involved in this type of memory, as an increased histamine concentration in the brain improved short-term recognition memory, whereas depletion of neuronal histamine had an amnesic effect (Prast et al. 1996). As previously mentioned, social recognition depends also on aging. The H_3 receptor antagonist ABT-239 that does not significantly improve social memory in adult rats improved recall in aged rats to the extent that their performance was comparable to that of adult rats, without altering exploratory behaviour (Fox et al. 2005). Other recently synthesized H_3 receptor antagonists were also found to enhance short-term memory in the rat social recognition memory model (Hudkins et al. 2014). Using a protocol entailing re-exposure of the adult rat to the same juvenile 90 min after the first encounter, Kraus and colleagues suggested that histaminergic neurotransmission within the nucleus accumbens facilitated short-term social memory without influencing cholinergic and glutamatergic transmission (Kraus, Prast and Philippu 2013). Another study used the social discrimination protocol to show that recognition consolidation is mediated by H_2 receptors in both the amygdala and dorsal hippocampus, as rats injected with the H_2 receptor antagonist ranitidine spent a similar length of time exploring the novel and familiar juveniles, and the H_2 receptor agonist dimaprit reversed this effect (Garrido Zinn et al. 2016). Nevertheless, H_2 receptor activation in the infralimbic cortex does not appear to participate in the consolidation of social recognition memory (Cavalcante et al. 2017). Kraus and colleagues had previously reported that an infusion of famotidine, another H_2 receptor antagonist, did not affect the thioperamide-induced facilitatory effect on recognition memory (Kraus et al. 2013). These apparently contrasting results could be related to differences in the injection site. Famotidine was administered into the brain ventricular system, whereas ranitidine was given directly into the BLA or CA1 at very similar dosages; thus, it is conceivable that the final concentration of famotidine within these specific structures was not sufficient to prevent thioperamide’s effects (Provensi et al. 2018a).

Interaction between the histaminergic and cholinergic system

Several evidences support the involvement of the cholinergic transmission as an essential neurophysiological component in cognitive functioning: (i) pharmacological experiments conducted in both animals and humans have shown learning and memory deficits after anticholinergic treatments, (ii) cholinergic disfunction and cell loss have been associated with memory alteration in aged subjects and Alzheimer’s disease patients, which has been related to cognitive deficits, (iii) the clinical efficacy of acetylcholinesterase inhibitors (galantamine,

donepezil, rivastigmine) for the cognitive deficits associated with mild to moderate Alzheimer's disease (Micheau and Marighetto 2011).

There are compelling reasons to believe that histamine modulates memory through interactions with other neurotransmitters regulated by H_3 receptors.

The report that in rats perfusion of the septum with H_3 receptor antagonists or H_2 receptor agonist, dimaprit, increased acetylcholine (ACh) release from the hippocampus, whereas R-α-methylhistamine, an H_3 agonist, produced the opposite effect (Bacciottini et al. 2002), evoked the intriguing hypothesis that histamine exerts this procognitive effect through the regulation of ACh release in the hippocampus. In this regard, it has been proposed that histamine participates in different associative learning tasks mainly by regulating the release of neurotransmitters such as ACh, dopamine and GABA (Passani et al. 2017). Passani and colleagues demonstrated that intra-BLA administration of H_3 receptor antagonists reduced both the freezing time of contextual fear conditioned rats and local ACh release (Passani et al. 2001); thus, amnesia may depend, at least in part, on the local modulation of the cholinergic tone. Consistently, H_3 receptor agonists given into the BLA ameliorated the expression of fear memory and increased the local release of ACh (Cangioli et al. 2002, Baldi and Bucherelli 2005).

H_3 receptor activation modulates ACh release from other brain regions as well, apparently with modalities that differ according to their cytoarchitectonics. Local H_3 receptor activation decreased ACh release from the cholinergic terminals in the neocortex (Blandina et al. 1996), through a neuronal arrangement that involves inhibition of GABAergic transmission (Giorgetti et al. 2000). Consistent with the reduction in cholinergic tone, stimulation of cortical H_3 receptors impaired performance in both passive avoidance and object recognition tasks (Blandina et al. 1996). Histamine-elicited decrease in cortical ACh release also has a significant impact on the formation of taste aversive memory, since an injection of R-α-methylhistamine, an H_3 agonist, into the insular cortex impaired conditioned taste aversive memory and simultaneously decreased the local release of ACh (Purón-Sierra and Miranda 2014). In this test, animals acquire an aversion to a specific taste when a gastric malaise follows its consumption. Inactivation of the cortex decreased the efficiency of taste aversive learning (Buresová and Bures 1974); cholinergic activity in the insular cortex is strongly implicated in the formation of taste mnemonic representation (Naor and Dudai 1996).

Other study demonstrated that injection of an acetylcholinesterase inhibitor, donepezil, or ABT-239 improved memory in the object recognition paradigm and augment GSK-3β

phosphorylation in cortical and hippocampal homogenates of wild type but not of acutely or chronically histamine-depleted animals (Provensi et al. 2016b).

Study of our laboratory demonstrated show that responses to H_3R antagonists differentiate histaminergic neurons according to their projection areas. When applied to the rat TMN, H_3R antagonists, such as thioperamide or GSK- 189254, invariably augmented histamine release from the TMN, from the prefrontal cortex and from the nucleus basalis magnocellularis (NBM), but not from the nucleus accumbens (NAcc), nor the striatum (Giannoni et al. 2009, Giannoni et al. 2010), demonstrating that H_3R 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 (Blandina et al. 2012).

Taken together, these observations strongly suggest a physiological role for the differential regulation, in a region-specific manner, of histamine and of neurotransmitters that are crucial modulators of memory processing and motivated behaviours. Therefore, the resulting effects of histaminergic ligands may depend on tissue-architectural constraints that separate groups of neurons in particular brain structures that modulate the expression of specific behaviours (Provensi et al. 2018b).

Aim of the study

During my PhD I focused my research mainly on the interplay between the brain histaminergic system and nutritional/hormonal interventions in lessening stress-induced maladaptive behaviours and memory impairments. I also investigated the role of the histaminergic system in sociability and the memory of social recognition.
For these reasons and for the purpose of clarity, the manuscript is divided in three sections.

In the first section of the thesis, I reported the results published in the publication Provensi et al., 2019 PNAS, that I co-authored and where I contributed to all experimental settings with the exception of microbiota analysis.
We evaluated the effects of a diet enriched with ω-3 polyunsaturated fatty acid (PUFA) and vitamin A in preventing immediate and long-lasting behavioural deficits and neurochemical changes induced by adolescent social instability stress, a well-validated animal model of social stress that produces long-lasting effects on cognitive and emotional responses that may persist for the entire lifespan (Burke et al. 2017, McCormick, Hodges and Simone 2015). These results prompt us to understand if the brain histaminergic system takes part in the protective effects of the enriched diet.

In the second part, I investigated whether the central histaminergic system mediates the preventive effect of the ω-3 PUFA and vitamin A enriched diet in stress-induced maladaptive behaviours and memory impairment. Indeed, brain histamine is very sensitive to stressful stimuli and our working hypothesis is that the histaminergic system is necessary to process signals from the periphery. We then evaluated the effects of the enriched diet in preventing behavioural, neurochemical and molecular changes induced by 10 days of chronic social defeat stress (CSDS) in mice genetically deprived of histamine ($HDC^{+/+}$) and wild type littermates ($HDC^{-/-}$). At the end of the stress period, the animals underwent a series of behavioural tests that are comprehensive of several domains affected by stress: social behaviour, mood, anxiety and cognition. Moreover, we analysed the hippocampus and prefrontal cortex composition in term of fatty acid metabolic enzymes by qPRC and synaptophysin, a synaptic vesicle glycoprotein that participate in synaptic transmission (Calhoun et al. 1996) measured by Western Blot analysis. To complete my metabolic studies, I joined the laboratory of Prof. Sophie Layé at University of Bordeaux in NutriNeuro Institute. Due to the pandemic emergency, I returned to Italy before all experimental settings were completed.

I also studied the interplay between the brain histaminergic system and the fatty acid derivative lipid Oleoylethanolamide (OEA) in preventing stress-induced deleterious effects, since our research group recently show that OEA require the central histaminergic system to exhibit its antidepressant-like effect . In this case we subjected $HDC^{+/+}$ and $HDC^{-/-}$ mice to 21 days of CSDS and based on the preliminary results published in the PhD thesis by Dr. Alessia Costa in 2017 that report an effect of OEA in preventing social aversion induced by chronic stress in $HDC^{+/+}$ but not $HDC^{-/-}$ mice, in collaboration with Dr. Andrea Santangelo we analysed the behavioural changes of mice subjected to CSDS and treated with OEA. The manuscript Rani et al., has been submitted to Neurobiology of Stress.

In the third part of my thesis I assessed the role of the histaminergic system in sociability and the memory of social recognition by using selective ligands of the histaminergic H_3 receptor on social behaviour and social memory. Indeed, the histaminergic system is strongly involved in the modulation of memory and of social recognition memory as well (Provensi et al. 2018b, Provensi et al. 2018a), however nowadays not much is known about the specific phases regulated by neuronal histamine. To investigate the impact of histaminergic neurotransmission deficiency or potentiation in short and long-term social recognition memory we subjected adult mice to the social recognition paradigm Reduction of brain histamine levels was achieved with 3 different approaches: (i) by using $HDC^{-/-}$ mice; (ii) mice infused with the HDC inhibitor α-Fluoromethylhistidine (a-FMH 1 mg/mL, 5mL) directly into the lateral ventricles and (iii) animals receiving a systemic injection of the H_3 receptor agonist VUF16839 (5 mg/kg, i.p.) which decreases histamine release. Increased histamine release was induced by systemic administration of the H_3R antagonist ciproxifan (3 mg/kg, i.p.). Part of the results are published in Wágner et al., J. Med Chem. 2020.

Part I: Preventing adolescent stress-induced cognitive changes by diet

In rodents, as in humans, adolescence is a time of developmental changes and reorganization in the brain and stress systems, marked by cognitive maturation and behavioural changes (Spear 2000b). ω-3 PUFAs play critical roles in the development and function of CNS. In this part of my thesis, we hypothesized that a diet enriched in ω-3 PUFAs and vitamin A may prevent immediate and long-lasting behavioural deficits and neurochemical changes induced by stress during adolescence.

Materials and Methods

Animals: Male Wistar rats obtained from Charles River (Lecco, Italy) were housed in the animal facility of Ce.S.A.L (Università di Firenze) upon arrival at 25 days of age. Rats were housed in pairs in a humidity and temperature-controlled room (22 ± 1°C) with a 12:12-h light-dark cycle (light on 07:00–19:00 h), Control or enriched diet (ssniff-Spezialdiäten GmbH, Germany) and water were available ad libitum except during the sucrose preference test. All procedures were conducted in accordance with the Council Directive of the European Community (2010/63/EU), with the Decreto Legislativo Italiano 26 (13/03/2014), and National Institutes of Health guidelines on animal care and approved by veterinarian supervision.

Diet composition: Diets were matched for macronutrient content; the specific compositions are provided in Table 1. To prevent oxidation of PUFAs, diets were maintained in air-sealed bags at 4 °C in the dark. Food was changed and weighed every day.

Table 1. Composition of the control diet and Omega 3 PUFA/Vit. A enriched diet (from Ssniff Spezialdiäten GmbH)

		Control Diet 5,000 IU/kg Vit. A SPP12-E020	EPA/DHA 45,000 IU/kg Vit. A S9912-E022
Casein	%	20.0000	20.0000
Corn Starch, pre-gelatinized	%	37.4984	37.4904
Maltodextrin	%	15.0000	15.0000
Sucrose	%	10.0000	10.0000
Cellulose	%	5.0000	5.0000
L-Cystine	%	0.3000	0.3000
Mineral & trace element premix	%	6.0000	6.0000
Vitamin premix, AIN *	%	1.0000	1.0000
TBHQ (t-butylhydroquinone)	%	0.0014	0.0014
Vitamin A	%	0.0002	0.0082
Choline Cl	%	0.2000	0.2000
HO Sunflower Oil	%	1.9000	1.4000
Palm Oil	%	2.2000	-
Canola Oil	%	0.8000	-
EPA oil	%	-	2.5000
Safflower oil	%	-	0.4000
Soybean Oil	%	0.1000	0.7000
Crude protein	%	17.6	17.6
Crude fat	%	5.1	5.1
Crude fibre	%	5.0	5.0
Crude ash	%	5.4	5.4
Starch	%	36.1	36.1
Sugar	%	11.1	11.1
Fatty Acids			
C 12:0	%	-	0.01
C 14:0	%	0.03	0.20
C 16:0	%	1.12	0.65
C 18:0	%	0.18	0.18
C 20:0	%	0.02	0.02
C 16:1	%	0.01	0.21
C 18:1	%	2.94	1.72
C 18:2	%	0.55	0.79
C 18:3	%	0.09	0.06
C 20:5 *n3*	%	-	0.44
C 22:5 *n3*	%	-	0.05
C 22:6 *n3*	%	-	0.30
Physiological fuel value	MJ/kg	15.5	15.5
Protein	kcal%	19	19
Fat	kcal%	13	13
Carbohydrates	kcal%	68	68

* Providing 4,000 IU/kg Vit. A

Social instability: Male Wistar rats arrived at our animal facility at PND 25 and were assigned at random to three experimental groups: non-stressed rats fed with the control diet (NSCD), rats subjected to social instability protocol and fed with the control diet (SCD), and stressed rats fed with the enriched diet (SED). The social instability stress involves changing the social housing conditions of adolescent rats, as described previously (McCormick et al. 2015). In brief, on each day from PND 30 to PND 45, the rats were isolated for 1 h in ventilated round plastic containers (10 cm in diameter), akin to restraint. After isolation, the rats were housed with a new partner undergoing the same procedure in a new cage. The stress regimen was implemented at various times during the light cycle to decrease the predictability of the event. After the last isolation on PND 45, the rats were returned to their original cage partners. The NSCD rats were not disturbed except for regular cage maintenance and weighing. The consequences of the social instability stress procedure were assessed during adolescence (PND 46–51) and during adulthood (PND 70–76) using a battery of tests comprehensive of several domains affected by chronic stress: cognition (novel object recognition and contextual fear conditioning), anhedonia-like behaviour (sucrose preference), and anxiety-like behaviour (elevated plus maze). Locomotor activity was measured in an open field arena. At 1 d after completion of the behavioural tests, hippocampus and prefrontal cortex were collected for neurochemical analyses. Different cohorts of animals were used at the two time points. The experimental timeline is depicted in Figure 10.

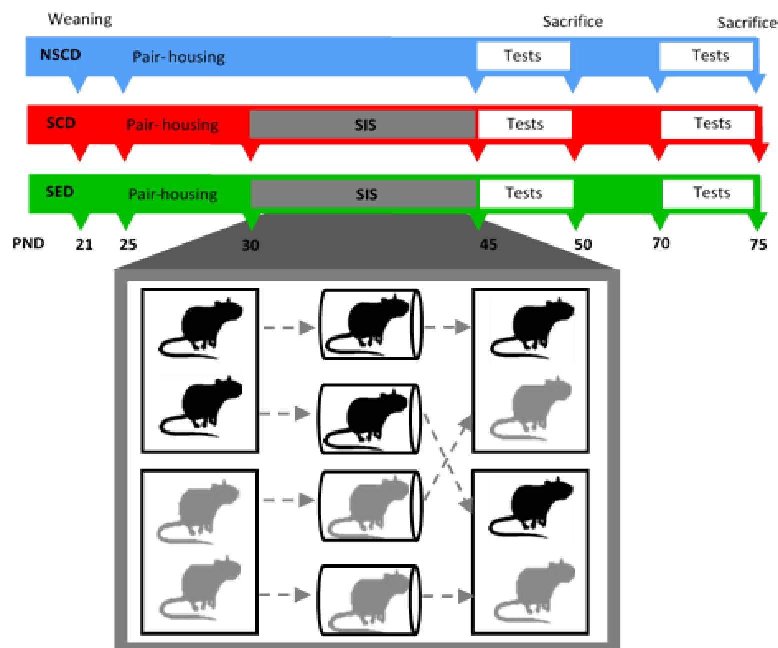


Figure 10. Time-line for the adolescent social instability stress experiment. Adolescent rats were randomly assigned to three experimental groups: NSCD=Non-stressed fed with control diet, SCD=Stressed fed with control diet, and SED=stressed fed with enriched diet (Provensi et al., 2019).

Novel Object Recognition Test: Rats' behaviour was assessed in an open-field arena (60 x 70 x 40 cm) placed in a sound attenuated room. The procedure for the NOR involved a total of three sessions: habituation, training and test separated by predetermined inter-trial intervals. Each animal was subjected to the procedure separately and care was taken to remove any olfactory/taste cues by cleaning carefully the arena and test objects between trials. During habituation rats freely explored the arena for 10 min. Twenty-four hours later, each rat was placed in the same position and facing the same direction into the test arena in the presence of two identical objects (plastic shapes such as cubes, cylinders or pyramids). The test session was performed 1 or 4 h after training, during which, each rat was again placed in the test arena for 5 min in the presence of one of the familiar objects and a novel object. Rats were placed in their home cages between trials. The position of the objects (left/right) was randomized to prevent bias from order or place preference. Animal's behaviour during all session was videotaped and the time spent actively exploring each object was recorded by an experienced observer unaware of the experimental groups. Exploration was defined as sniffing or touching the stimulus object with the nose and/or forepaws. Sitting on or turning around the objects was not considered exploratory behaviour. The final data is expressed as the percentage of time exploring the familiar and new objects during the test.

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. 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 2 min and subsequently 3 electric footshocks (2s, 0.5 mA) were delivered at 30s intervals. The footshock intensity was chosen according to previous published data from our laboratory (Provensi et al. 2017). Rats were removed from the chamber 1 min after the last footshock and placed back in their home cages. Twenty-four hours after conditioning, rats were again placed inside the conditioning apparatus in the soundproof room and left undisturbed for 3 min. Freezing, the complete absence of somatic motility with the exception of respiratory movements, was measured with a stopwatch by personnel unaware of the experimental group. Results are expressed as the time the animals spent freezing (in seconds) during each session which is considered an index of contextual fear learning. Each rat was tested either during adolescence or during adulthood.

Sucrose preference test: To assess anhedonia-like behaviour, rats were allowed to habituate to the 1% sucrose solution and the two-bottle procedure by introducing two identical bottles to their home cage for 24 hs, one containing 1% sucrose and the other tap water. After acclimatization, sucrose preference was evaluated at three time points: (i) at PND29 prior to social instability procedure to obtain a baseline measure of sucrose preference, (ii) at PND45 the end of stress procedure (adolescence) and (iii) during adulthood at PND70. To test sucrose preference, the rats were deprived of food and water for 5 hours, then presented with two bottles, one containing 1% sucrose and the other tap water. The test lasted 1 h beginning at the start of the dark-phase (19:00-20:00 h). All bottles were weighed before and after the test, and results were expressed as percentage of sweetened or water consumption. The right-left placement of the sucrose and water bottles were counter-balanced for all animals in test days.

Elevated Plus Maze: The apparatus consisted of two open (50 x 10 cm) and two closed (50 x 10 X 37 cm) arms extending from a central platform (10 X 10 cm) elevated 50 cm above the floor. Each rat was placed in the central platform of the maze, facing the open arm opposite to the experimenter, and videotaped test session of 5 min duration for each trial. Observers blind to experimental groups measured the amount of time spent in the open and closed arms, as well as the number of open and closed arm entries. Between tests, the apparatus was cleaned with a 30% ethanol solution in water and was allowed to dry thoroughly.

Open field: The open-field apparatus used in this experiment consisted of a 60 x 70 x 40 cm square arena. Rats were placed facing the centre of the arena and allowed to freely explore for 10 minutes. They were then returned to the home cage after the test. After each observation, the arena was cleaned with 30 % ethyl alcohol in water to remove possible scent cues left by the animal. For analysis, the space of the arena was virtually divided into a central square (25%) and a peripheral zone (75%) and several behavioural parameters were investigated using Smart 2.5 software. Exploratory behaviours such as rearing, grooming and climbing were recorded by trained observers unaware of the experimental groups.

Western blot analysis: For the neurochemical determinations, rats were sacrificed 24 hs after the end of behavioural evaluations. After sacrifice, rat brains were dissected out on ice and cortices and hippocampi immediately isolated. The pooled structures (left and right) were individually homogenized in 400 µL ice-cold lysis buffer containing protease and phosphatase inhibitors (50mM TrisHCl (pH 7.5), 50mM NaCl, 10mM EGTA, 5mM EDTA, 2mM NaPP,

4mM PNFF, 1mM Na3VO4, 1.1mM PMSF, 20 µg/µL Leupeptin, 50 µg/µL Aprotinin, 0.1% SDS) and centrifuged at 12000 rpm at 4 °C for 15 minutes. The supernatant was collected and total protein levels were quantified using the Pierce BCA Protein Assay (Thermo Scientific, USA). Homogenates were diluted in a mix of lysis buffer and loading buffer 2x (50mM Tris pH = 6.8, 100mM DTT, 10% Glycerol, 1% Bromophenol Blue, and 2% SDS) and boiled for 10 minutes. Aliquots containing 40 µg or 10 µg of total proteins, for detection of BDNF or synaptophysin respectively, were resolved by electrophoresis on a 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon Transfer Membranes, Millipore, USA). Blots were blocked in Tris-buffered saline, pH 7.6 containing 0.1% of Tween 20 (TBS-T) and 5% non-fat dry milk (Bio-Rad Laboratories, USA) for 2 h at room temperature and then incubated overnight at 4°C with antibodies against BDNF (1:1000 Abcam), synaptophysin (1:10000 ThermoFisher Scientific) or tubulin (1:1000 Cell Signaling) all diluted TBS-T containing 5% non-fat dry milk. Immunodetection was performed with secondary antibodies (anti-rabbit IgG conjugated to horseradish peroxidase, Cell Signaling technology, USA) diluted 1:5000 in TBS-T containing 1% of non-fat dry milk. Membranes were washed in TBS-T and then reactive bands were detected using enhanced chemiluminescence (Luminata Crescendo, Millipore, USA). Quantitative densitometric analysis was performed using the QuantityOne analysis software (Bio-Rad). For each sample, a ratio of BNDF/Tubulin or Synaptophysin/Tubulin densities was calculated and then all the individual rates were expressed as a percentage of the average of ratios obtained from control groups.

Statistical analysis: Data were analysed using Graphpad Software (version 6.0). The data presented in graphs or tables are expressed as mean and S.E.M. or mean ± S.D., respectively. Statistical significance was determined using One- or Two-way ANOVA followed by Bonferroni's or Newman-Keuls multiple comparison post-hoc tests. The level of significance was set to P<0.05.

Results

Effects of Stress and the Enriched Diet on Body Weight and Food Consumption.

As shown in Figure 11A, adolescent SCD gained less weight than NSCD ($F_{(2,61)}$= 9.950; P < 0.001], an effect that persisted until adulthood ($F_{(2,59)}$= 5.262; P < 0.01) (Figure 11B). This effect was counteracted by the ω-3 PUFA/vitamin A–enriched diet. At both ages, rats ate comparable amounts of food independent of stress and diet (Figure 11C-D).

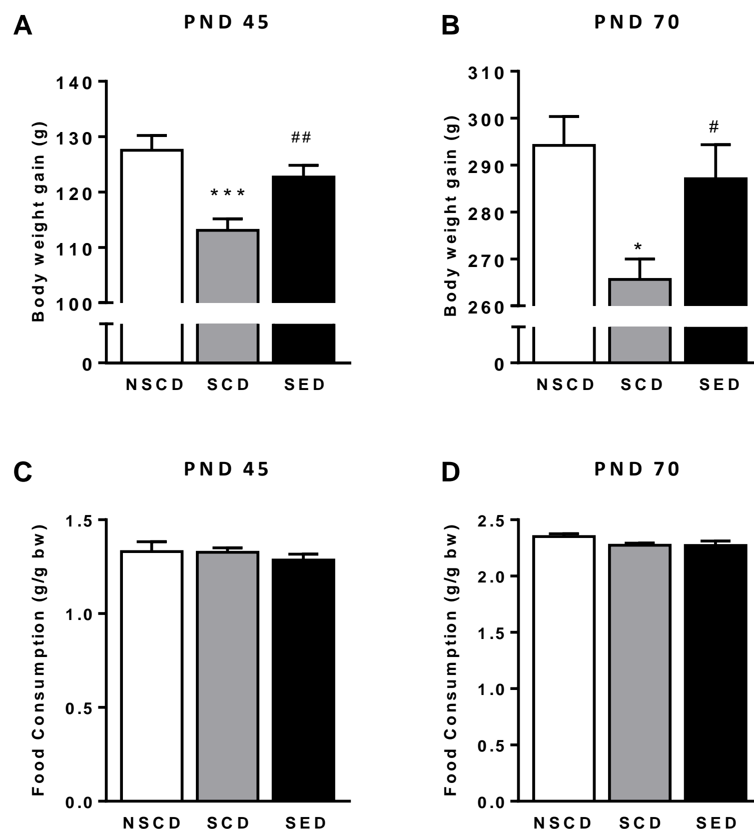


Figure 11. (A-B) Impact of social instability stress and diet enrichment on body weight and cumulative food consumption measured during adolescence (at post-natal day 45, A) and at completion of the stress procedure during adulthood (at post-natal day 70, B). n = 18–24 rats/group. ***P < 0.001, *P < 0.05 vs. NSCD rats; $^{\#\#}$P < 0.01, $^{\#}$P < 0.05 vs. NSCD by one-way ANOVA and the Bonferroni test (Modified from Provensi et al., 2019).

The Enriched Diet Prevented the Recognition memory Impairments Induced by Social Instability Stress

The Enriched Diet Prevented the Cognitive Impairments Induced by Social Instability Stress when testes in Novel Object Recognition task. Two weeks of social instability stress had a negative impact on recognition memory that persisted into adulthood. For short-term memory (i.e., at 1 h after training), adolescent rats spent significantly more time exploring the novel object regardless of stress or diet [objects: $F(1,34) = 62.88$, $P < 0.001$; condition: $F(2,34) = 0.0$, $P > 0.05$; interaction: $F(2,34) = 4.43$, $P > 0.05$] (Figure 12A). Regarding long-term memory (i.e., at 4 h after training), SCD rats did not discriminate between the two objects (Figure 12B). However, in SED rats, the enriched diet fully prevented the stress-induced impairment of object discrimination [objects: $F(1,30) = 59.11$, $P < 0.001$; condition: $F(2,30) = 0$, $P > 0.05$; interaction: $F(2,30) = 15.01$, $P < 0.01$]. The cognitive impairment induced by the social instability persisted into adulthood and was prevented by the enriched diet administered since adolescence. Indeed, when tested in adulthood, SED rats showed a total prevention of the memory impairment induced by adolescent stress [objects: $F(1,30) = 67.45$, $P < 0.001$; conditions: $F(2,30) = 0$, $P > 0.05$; interaction: $F(2,30) = 14.08$, $P < 0.001$] (Figure 12D).

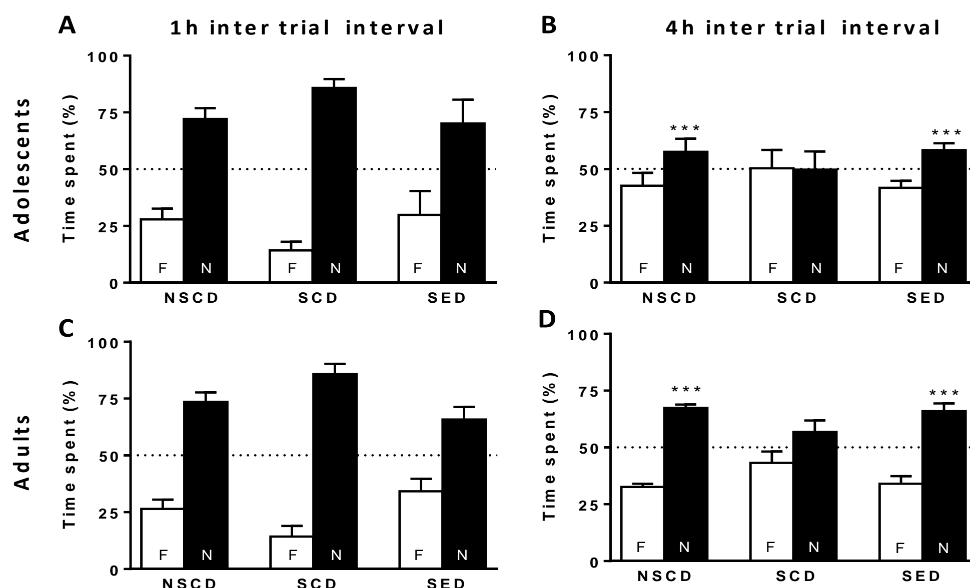


Figure 12. The enriched diet prevented stress-induced cognitive impairment in the novel object recognition test. (A and C) Stress did not affect the performance of either adolescent or adult rats when the test was performed at 1 h after training. (B and D) Adolescent and adult stressed rats showed memory impairment when tested at 4 h after training, which was prevented by dietary supplementation with ω-3 PUFA/vitamin A. n = 6–8 rats/group. ***P < 0.001; **P < 0.01; *P < 0.05 vs. familiar object within each experimental group by two-way ANOVA and the Bonferroni test. (Modified from Provensi et al., 2019)

The Enriched Diet Prevented the Emotional memory Impairments Induced by Social Instability Stress

We also tested rats with an emotional arousing training experience that engages both contextual and emotional memory processing: the contextual fear conditioning paradigm. Freezing time obtained during a 3-min reexposure of rats to the conditioning apparatus at 24 h after acquisition served as an index of memory of the aversive experience. As shown in Figure 13A, social instability stress did not affect the acquisition of fear memory irrespective of diet, whereas SCD adolescent rats froze less during context retrieval compared with non-stressed adolescent rats fed with control diet [NSCD, $F_{(5,55)} = 22.77$, $P < 0.001$]. The enriched diet restored contextual fear memory expression, as SED rats spent significantly more time freezing at recall compared with SCD rats, which were indistinguishable from NSCD rats. The emotional memory deficit and beneficial effects of the enriched diet were long-lasting, as rats tested in adulthood showed a comparable behaviour as the adolescents. SED adult rats froze for a significantly longer time than SCD rats [$F_{(5,55)} = 47.95$, $P < 0.001$] (Figure 13B).

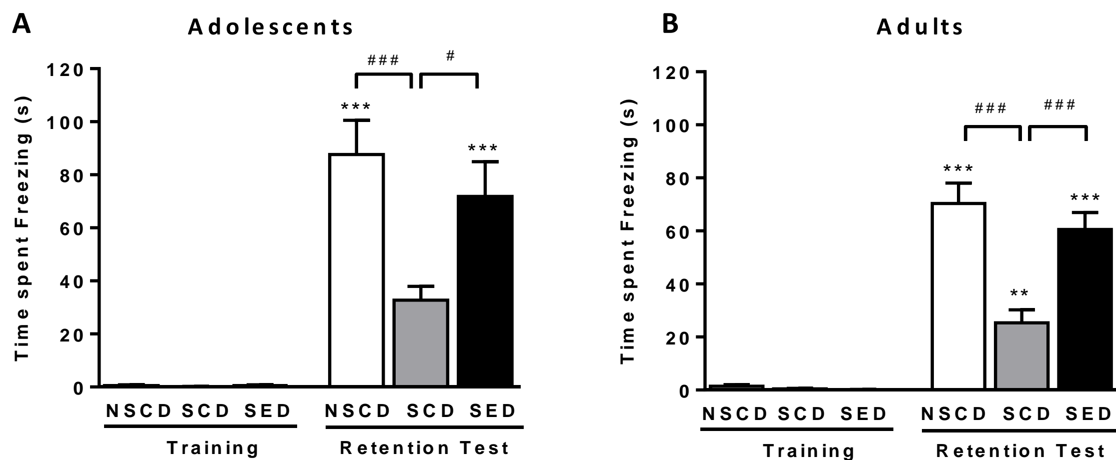


Figure 13. The enriched diet prevented immediate (A) and long-term (B) stress-induced cognitive impairment in the contextual fear conditioning test. Rat freezing time did not differ at training regardless of treatment condition. When tested at 24 h after training, the SCD rats showed a lower freezing time than the NSCD rats, and the SED rats showed no stress-induced cognitive impairment. n = 9–10 rats/group. ***P < 0.001, **P < 0.01 vs. respective training; ###P < 0.001, #P < 0.05 vs. SCD by one-way ANOVA and the Bonferroni test (Modified from Provensi et al., 2019).

Stress and diet did not affect anhedonia-like behaviour

In agreement with recent data (Marcolin et al. 2019), we observed no differences between SCD and NSCD rats in terms of preference for the consumption of a sweetened solution when evaluated at different time points (Figure 14). Neither stress nor the enriched diet had an effect on anhedonia-like behaviour, as the preference for a sucrose-sweetened drink was not affected by any of the experimental manipulations in either age group tested.

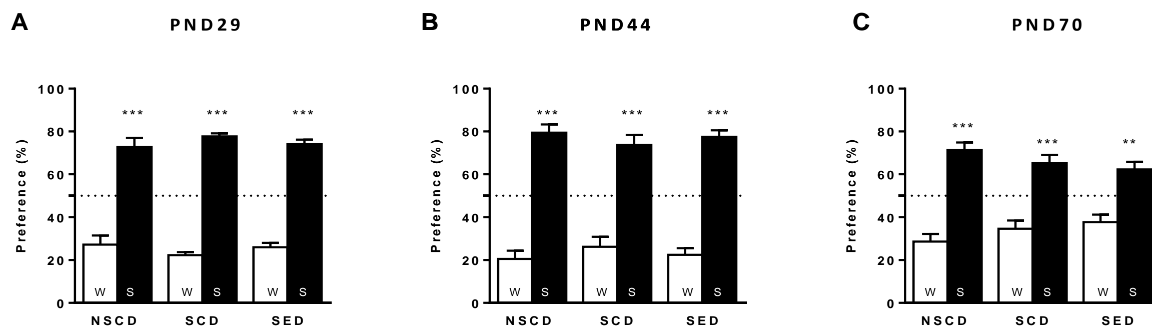


Figure 14. Social instability stress did not induce an anhedonia-like state. Consumption of a sweetened solution (sucrose 1%, S) or tap water (W) during 1h were measured at 3 time points: (A) PND 29 in order to a baseline measure; (B) PND 44, immediately at the end of stress procedure (adolescence) and (C) PND70, during adulthood. Preference was calculated as the percentage of sucrose solution over tap water consumption. NSCD stressed-control diet, SCD stressed control diet; SED stressed enriched diet; W, water; S, sucrose. ***P<0.001; **P<0.01 vs water consumption within the same experimental group by two-way ANOVA and Bonferroni's test. Shown are means ± SEM of 18-24 animals per experimental group. (Modified from Provensi et al., 2019)

Neither stress or diet affected locomotion or anxiety-like behaviour.

Locomotor activity, measured as the distance travelled and time spent moving in an open field, was comparable across the three cohorts in each age group, although adult rats were less active than adolescents (Table 2). Neither stress nor diet affected the number of entries and time spent in the centre or the periphery of the open field (Table 2).

Treatment conditions did not affect other exploratory behaviours indicative of a non-distressed state, such as climbing, rearing, and grooming. Accordingly, treatment conditions also had no effect on the number of entries and percentage of time spent by adolescent or adult rats in the open arms of the elevated plus maze (Table 3).

Table 2. Social instability stress did not alter locomotor activity and exploratory behaviours measured in an open field arena at any age. NSCD stressed-control diet SCD stressed control diet; SED stressed enriched diet. Results are expressed as means ± SD of 10 animals per experimental group. F ratios and P values were determined using one-way ANOVA.

	Parameter	Zone	NSCD	SCD	SED	F ratios	P value
Adolescents	Entries	Central	11.7 ± 4.1	12.1 ± 6.1	14.0 ± 3.8	$F_{(2,29)}$= 0.669	0.520
	Time spent (s)	Central	36.2 ± 13.1	47.8 ± 18.3	41.7 ± 23.7	$F_{(2,29)}$= 0.996	0.397
		Peripheral	563.7 ± 13.0	551.1 ± 16.2	558.1 ± 23.7	$F_{(2,29)}$= 1.201	0.316
	Distance travelled (cm)	Central	201.9 ± 78.4	205.3 ± 86.7	208.2 ± 57.3	$F_{(2,29)}$= 0.002	0.982
		Peripheral	1959.7 ± 352.9	1833.4 ± 581.1	2060.9 ± 314.4	$F_{(2,29)}$= 0.694	0.508
		Total	2161.6 ± 418.6	2038.7 ± 679.7	2269.1 ± 286.3	$F_{(2,29)}$= 0.587	0.563
	Climbings		18.4 ± 7.1	19.6 ± 5.5	23.8 ± 8.6	$F_{(2,29)}$= 1.466	0.249
	Rearings		8.7 ± 7.2	5.8 ± 7.0	9.8 ± 5.9	$F_{(2,29)}$= 0.939	0.403
	Groomings		2.1 ± 0.9	1.5 ± 1.3	1.9 ± 1.0	$F_{(2,29)}$= 0.8317	0.446
	Time spent Moving (s)	Central	9.2 ± 4.6	9.5 ± 5.8	10.5 ± 2.4	$F_{(2,29)}$= 0.228	0.798
		Peripheral	77.0 ± 29.6	73.6 ± 36.7	89.0 ± 28.0	$F_{(2,29)}$= 0.654	0.528
		Total	86.2 ± 33.6	83.0 ± 42.1	99.5 ± 27.7	$F_{(2,29)}$= 0.621	0.621
Adults	Entries	Central	7.5 ± 4.1	8.3 ± 4.2	7.6 ± 7.5	$F_{(2,29)}$= 0.104	0.902
	Time spent (s)	Central	27.2 ± 22.5	26.9 ± 14.8	21.7 ± 15.3	$F_{(2,29)}$= 0.300	0.743
		Peripheral	572.6 ± 22.5	572.9 ± 14.8	578.1 ± 15.3	$F_{(2,29)}$= 0.303	0.741
	Distance travelled (cm)	Central	131.7 ± 86.3	109.9 ± 48.1	125.8 ± 65.9	$F_{(2,29)}$= 0.271	0.764
		Peripheral	1633.5 ± 235.8	1669.8 ± 494.6	1481.6 ± 190.6	$F_{(2,29)}$= 0.889	0.423
		Total	1765.2 ± 240.6	1779.7 ± 527.2	1607.4 ± 239.2	$F_{(2,29)}$= 0.698	0.507
	Climbings		16.7 ± 4.7	16.2 ± 9.4	15.8 ± 3.9	$F_{(2,29)}$= 0.049	0.953
	Rearings		5.2 ± 3.6	1.8 ± 2.0	1.9 ± 2.0	$F_{(2,29)}$= 0.776	0.146
	Groomings		3.4 ± 2.1	0.9 ± 1.3	1.1 ± 1.2	$F_{(2,29)}$= 0.370	0.694
	Time spent Moving (s)	Central	5.8 ± 3.7	6.5 ± 3.9	8.0 ± 5.1	$F_{(2,29)}$= 0.684	0.523
		Peripheral	51.3 ± 23.3	59.6 ± 24.4	69.0 ± 22.5	$F_{(2,29)}$= 1.433	0.256
		Total	57.1 ± 25.2	66.1 ± 28.2	77.0 ± 26.4	$F_{(2,29)}$= 1.402	0.263

Table 3. Social instability stress did not alter anxiety-related behaviours evaluated in the elevated plus maze test

	Parameter	Zone	NSCD	SCD	SED	F ratios	P value
Adolescents	Time spent (s)	Open Arms	38.2 ± 35.1	32.5 ± 17.8	38.8 ± 17.8	$F_{(2,29)}$= 0.192	0.826
		Closed Arms	171.2 ± 47.3	157.7 ± 30.4	156.4 ± 24.2	$F_{(2,29)}$= 0.537	0.590
		Total	209.3 ± 37.2	190.2 ± 27.4	195.2 ± 32.1	$F_{(2,29)}$= 0.930	0.407
	Entries	Open Arms	3.4 ± 2.0	3.2 ± 1.9	4.3 ± 2.1	$F_{(2,29)}$= 0.874	0.429
		Closed Arms	9.0 ± 3.8	10.3 ± 3.0	11.1 ± 2.7	$F_{(2,29)}$= 1.103	0.346
		Total	12.4 ± 3.8	13.5 ± 3.7	15.4 ± 3.7	$F_{(2,29)}$= 1.640	0.213
	Time spent (%)	Open Arms	18.3 ± 15.4	17.3 ± 9.3	19.5 ± 6.9	$F_{(2,29)}$= 0.098	0.907
		Closed Arms	81.7 ± 15.4	82.7 ± 9.3	80.5 ± 6.9	$F_{(2,29)}$= 0.098	0.907
	Entries (%)	Open Arms	28.2 ± 14.0	23.3 ± 11.0	27.2 ± 9.3	$F_{(2,29)}$= 0.515	0.634
		Closed Arms	71.8 ± 14.0	76.7 ± 11.0	27.2 ± 72.8	$F_{(2,29)}$= 0.515	0.634
Adults	Time spent (s)	Open Arms	22.0 ± 18.6	26.2 ± 19.2	20.8 ± 11.8	$F_{(2,29)}$= 0.298	0.758
		Closed Arms	209.9 ± 38.8	178.8 ± 31.0	182.0 ± 25.0	$F_{(2,29)}$= 2.826	0.076
		Total	231.8 ± 35.4	205.0 ± 25.1	202.9 ± 24.8	$F_{(2,29)}$= 3.135	0.069
	Entries	Open Arms	2.2 ± 2.2	2.7 ± 2.5	2.8 ± 1.8	$F_{(2,29)}$= 0.143	0.867
		Closed Arms	7.5 ± 4.1	10.9 ± 2.8	8.1 ± 3.5	$F_{(2,29)}$= 2.644	0.089
		Total	9.7 ± 5.3	13.6 ± 4.5	10.4 ± 4.7	$F_{(2,29)}$= 1.850	0.177
	Time spent (%)	Open Arms	9.6 ± 8.6	12.9 ± 9.2	10.3 ± 6.0	$F_{(2,29)}$= 0.466	0.633
		Closed Arms	90.4 ± 8.6	87.1 ± 9.2	89.7 ± 6.0	$F_{(2,29)}$= 0.466	0.633
	Entries (%)	Open Arms	23.1 ± 76.9	17.4 ± 13.2	22.7 ± 12.6	$F_{(2,29)}$= 0.472	0.629
		Closed Arms	76.9 ± 17.5	82.3 ± 13.2	77.3 ± 12.6	$F_{(2,29)}$= 0.472	0.629

The Enriched Diet Prevented Short- and Long-Term Social Stress-induced effects on BDNF Expression.

To investigate the effect of stress and diet on brain plasticity, we assessed the expression of BDNF in the brain. We found significant differences in BDNF protein levels in the hippocampus of adolescent rats [$F_{(2,27)}$= 10.33, P < 0.001; Figure 15A]. BDNF expression was decreased in SCD rats compared with NSCD rats, and this effect was prevented by the enriched diet. In the frontal cortex of adolescent rats, stress did not significantly modify BDNF expression, whereas the enriched diet increased BDNF levels [$F_{(2,27)}$= 5.808, P < 0.01] (Figure 15B). Interestingly, social instability stress led to a long-lasting decrease in hippocampal BDNF expression, whereas the enriched diet prevented the effects of social instability stress [$F_{(2,28)}$ = 6.896, P < 0.01] (Figure 15C). BDNF levels in the frontal cortex were significantly increased following the enriched diet in adult rats as well [$F_{(2,24)}$ = 3.680, P < 0.05] (Figure 15D).

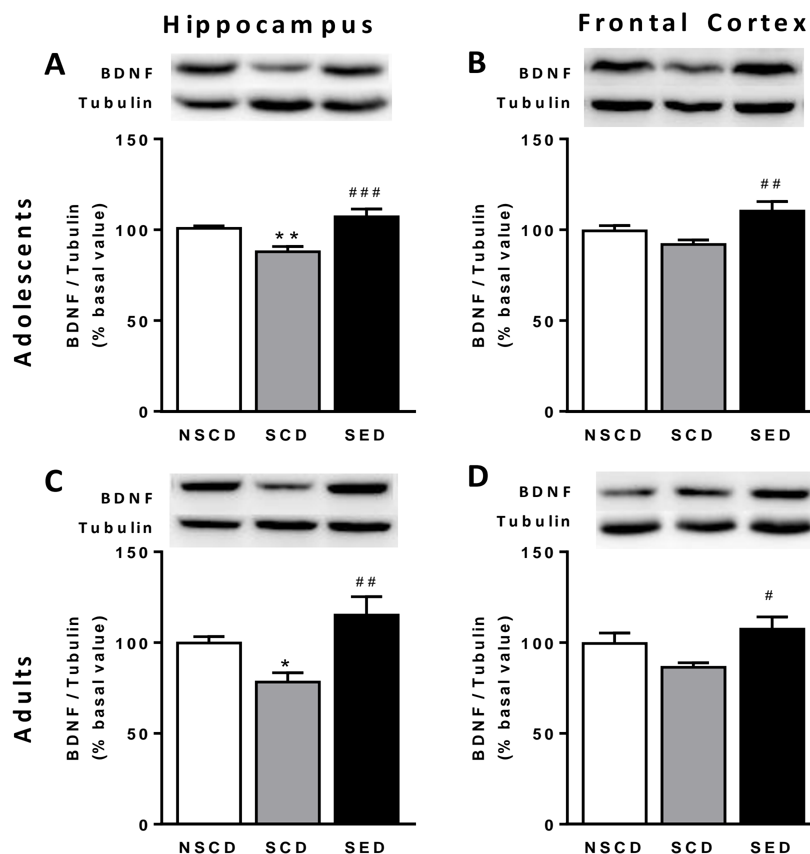


Figure 15. The enriched diet restored BDNF expression in the brain of stressed rats. (A and B) Stress decreased BDNF levels in the hippocampus in both adolescent (A) and adult (B) rats. The enriched diet restored BDNF expression to control levels. (C and D) In the prefrontal cortex of stressed rats, the BDNF decrease did not reach statistical significance in either adolescence (C) or adulthood (D); nonetheless, the enriched diet augmented BDNF expression compared with stressed and control rats. (Insets) Representative immunoblots for each experimental group. n = 8–10 rats/group. **P < 0.01; *P < 0.05 vs. NSCD; ###P < 0.001, ##P < 0.01, #P < 0.05 vs. SCD by one-way ANOVA and Bonferroni's test. (Modified from Provensi et al., 2019)

Enriched diet increases synaptophysin expression in the brain of stressed rat

We also used Western blot analysis to detect synaptophysin (a glycoprotein associated with presynaptic vesicles) as a marker of synaptic density. Synaptophysin expression was not significantly affected by stress in both adolescent and adult rats (Figure 16), in agreement with a previous report (McCormick et al. 2012). In adolescent rats, the enriched diet did not significantly affect synaptophysin expression in the hippocampus [$F_{(2,28)}$= 2.121, P > 0.05] or in the cortex [$F_{(2,29)}$= 2.727, P > 0.05]. In adult rats, however, we observed a significant diet-induced increase of synaptophysin expression in both the hippocampus [$F_{(2,26)}$= 8.858, P < 0.001] and the frontal cortex [$F_{(2,27)}$= 3.705, P < 0.05], which is consistent with the previous observation that long exposure to ω-3 PUFAs increases hippocampal synaptophysin expression (Venna et al. 2009)

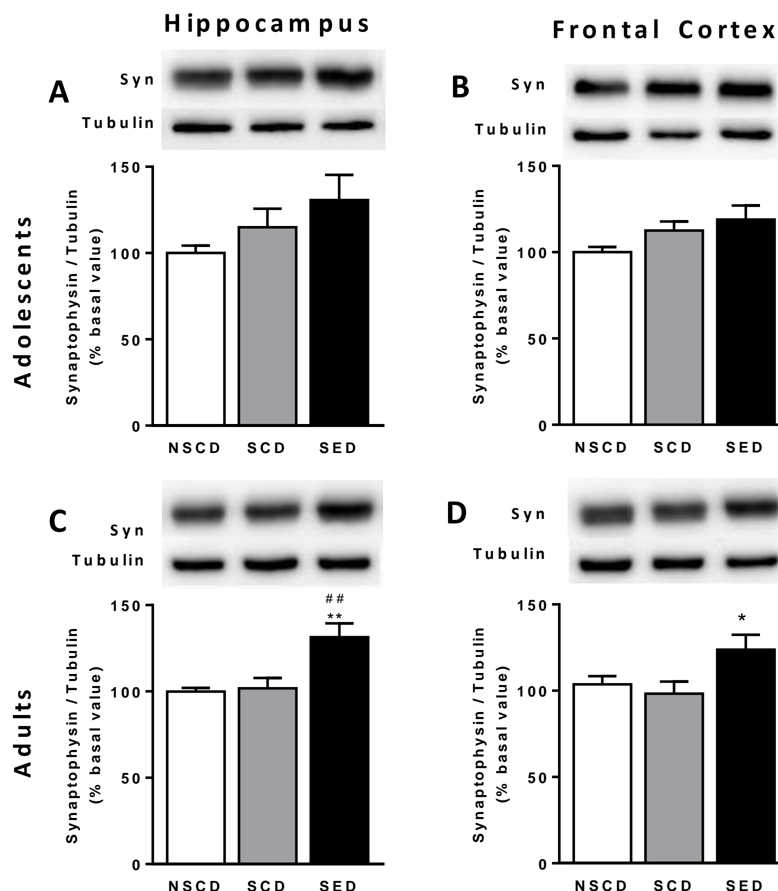


Figure 16. Representative immunoblots and densitometric quantification of synaptophysin in hippocampal (A,C) and cortical (B,D) homogenates from the different experimental groups. Stressful manipulation did not alter synaptophysin expression in both structures at any age. The enriched increased synaptophysin expression in hippocampus (C) as well as in the frontal cortex (D) of adult rats. NSCD stressed-control diet, SCD stressed control diet, SED stressed enriched diet. **P<0.01; *P<0.05 vs NSCD; ###P<0.001, ##P<0.01, #P<0.05 vs SCD by ANOVA and Bonferroni's test. Shown are means ± SEM of 9-10 animals per experimental group. (Modified from Provensi et al., 2019)

Conclusions: Part I

Nutrition has a fundamental role in maintaining brain health and behaviour at critical periods, especially in adolescence (Hueston et al. 2017). In rodents, appropriate essential micronutrient supplementation protects against the cognitive decline associated with early life stress (Naninck et al. 2015). Our study corroborates the idea that dietary intervention affects neurobehavioral development (Robertson et al. 2017) by demonstrating that a diet supplemented with ω-3 PUFAs and vitamin A prevented the deleterious cognitive decline induced by social instability stress during adolescence, with the amelioration maintained in adulthood. Social instability stress during adolescence is known to cause emotional and recognition memory impairments that persist into adulthood. These behavioural changes are closely associated with alterations in BDNF expression in the hippocampus and the frontal cortex. In the present study, dietary ω-3 PUFA/vitamin A exposure from adolescence to adulthood was sufficient to prevent such alterations, and the beneficial outcomes were maintained throughout adulthood. The rationale for using a combination of ω-3 PUFAs and vitamin A stems from recent findings demonstrating a beneficial synergistic effect of vitamin A and EPA/DHA on behavioural and neurobiological markers in aged rats (Létondor et al. 2016). Multiple levels of interactions occur between ω-3 PUFAs and retinoid signalling, because retinoic acid (the active metabolite of vitamin A) and DHA may bind to common nuclear receptors (Lane and Bailey, 2005; Su, 2010). Indeed, DHA and Vitamin A can bind to nuclear receptors, such as the PPARs (Evans and Mangelsdorf, 2014) and they activate kinase signalling pathways such as, AKT or the MAPK, which includes ERK1/2, (Masia et al., 2007; Rao et al., 2007; Al Tanoury et al., 2013) that are involved in the modulation of cerebral plasticity and thus in learning and memory processes (Giese and Mizuno, 2013).

Evidence indicates that there is a close relationship between ω-3 PUFA and vitamin A signalling pathways with both intra- and extra-nuclear interactions (Létondor et al., 2016), suggesting that these nutrients may act together to modulate synaptic plasticity processes and memory altered.

Social instability stress in adolescence exerts long-lasting effects on aversive and recognition memory, as shown in previous reports describing enduring deficits in contextual fear memory in response to adolescence stress (Morrissey, Mathews and McCormick 2011). The hippocampus is one of the brain structures crucially involved in regulation of stress responses (Reul and de Kloet 1985). We found that social instability impaired contextual fear memory, a predominantly hippocampus-dependent form of aversive memory (reviewed in ref. (Kim and

Diamond 2002)), in both adolescent and adult rats. In addition, stressed adolescent rats showed long-lasting memory impairment in the novel object recognition test, an effect not previously observed. We found a close correspondence between rats' memory performance and BDNF expression in the hippocampus, as both effects were significantly decreased in stressed adolescent and adult rats, and the enriched diet prevented both effects. Our results are in agreement with recent observations that ω-3 PUFAs induce increased BDNF expression in rat hippocampus (Vines et al. 2012). Unequivocal evidence suggests a key role for BDNF in the initiation of fear memory consolidation. BDNF enhances fear memory, and antibodies against BDNF impair fear memory when administered into the CA1 region of the hippocampus (reviewed in ref. (Izquierdo et al. 2016)). Our behavioural results are in accordance with these observations, as social instability stress reduced hippocampal BDNF expression and impaired contextual fear memory.

In the frontal cortex, BDNF expression is required for fear memory consolidation and expression (reviewed in ref. (Bekinschtein, Cammarota and Medina 2014)). Social instability stress did not significantly affect BDNF expression levels in the cortex of adolescent and adult rats, although the enriched diet augmented BDNF levels, presumably contributing to maintenance of long-term memory.

Social instability during adolescence is known to modify several social behaviours, as stressed rats spend less time in social interactions with other males, have reduced sexual performance, and exhibit longer latency to enter the centre of an open arena (Green, Barnes and McCormick 2013), all validated measures of anxiety-like behaviour. Furthermore, the modified social repertoire is evident in adulthood even weeks after the stressful procedure (Burke et al. 2017). In our study, though, we found no difference between stressed and non-stressed rats in the latency to enter the centre of the arena or the number of entries, or in behavioural differences in the elevated plus maze during adolescence or adulthood. One factor contributing to these discrepancies may be strain differences, which are known to be responsible for the anxiety profile (Ramos et al. 1997). Indeed, the Wistar rats used in our experiments appeared more resilient than those commonly used for anxiety-like tests. Other behavioural signs of stress, such as modified grooming, rearing, or climbing (Kruk et al. 1998, Füzesi et al. 2016) were not affected by our protocol or by the dietary supplementation. Confirming recent data regarding the consumption of natural rewards (Marcolin et al. 2019), neither adolescent nor adult stressed rats manifested anhedonia-like behaviour in the sucrose preference test.

Part IIa: Role of brain histamine in the effects of a diet to prevent social defeat stress-induced cognitive and neuropsychologic modifications

ω-3 PUFA supplementation increased synaptic plasticity in the hippocampus and improved memory in rodents under stress, but it may show little effect under normal conditions (Joffre et al. 2014, Bazinet and Layé 2014). Altered PUFAs metabolism has been reported to be involved in different neurological disorders via sustained neuroinflammatory processes (Joffre et al. 2014). Here we propose that a ω-3 PUFA and vitamin-A supplemented diet confers resilience to stress-induced maladaptive behaviours in a model of stress by activating the histaminergic system which is crucial in controlling arousal and cognition and is profoundly affected by stress (Haas et al. 2008). Therefore, we investigated its involvement in the effects produced by chronic stress and dietary supplementation with ω-3 PUFA/Vitamin-A in mice.

Material and Methods

Animals: Histidine decarboxylase null (C57bl/6, $HDC^{-/-}$) and wild type (C57bl/6, $HDC^{+/+}$) mice were grown in the Centro Stabulazione Animali di Laboratorio (CeSAL), Università di Firenze in humidity, temperature (22 - 24 °C) and light (light on 7:00-19:00)-controlled room. Mice were allowed free access to food and water. Genotypes were confirmed using the PCR protocol according to (Provensi et al. 2014). At postnatal (PND) day 21, mice were weaned and fed with Control or enriched diet (ssniff-Spezialdiäten GmbH, Germany). Nine to 13-week-old male CD1 retired breeders (Charles River, Italy) fed with standard diet (Mucedola s.r.l., Milan, Italy) were screened for aggressive behaviour and used for the social defeat stress protocol according to (Golden et al. 2011). All experiments were performed in accordance with the EEC recommendations for the care and use of laboratory animals (2010/63/EU) and approved by the Animal Care Committee of the University of Florence and Italian Ministry of Health (authorization n. 114-2017PR) and complying to the 3R. Ethical policy of the Università di Firenze complies with the Guide for the Care and Use of Laboratory Animals of the Council Directive of the European Community (2010/63/EU) and the Italian Decreto Legislativo 26 (13/03/2014). Every effort was made to minimize animal suffering and to reduce the number of animals used. All animals were weighted, and food consumption calculated daily.

Diet composition: Diets were matched for macronutrient content; the specific compositions are provided in Table 1. To prevent oxidation of PUFAs, diets were maintained in air-sealed bags at 4 °C in the dark. Food was changed and weighed every day.

Chronic Social Defeat Stress: C57bl/6 mice were singly housed prior to undergoing social defeat stress. CD1 mice were used as resident aggressors for the social defeat stress and were singly-housed prior to the experiments. Aggressive CD-1 mice, as defined by demonstrating at least one successful act of aggression during two consecutive days toward another male CD1 intruder mouse, were selected for use in the social defeat. A group of $HDC^{+/+}$ and of $HDC^{-/-}$ mice fed with control or supplemented diet were subjected to the CSDS protocol for 10 days (PND56 to PND76); adapted from (Golden et al. 2011). Briefly, the procedure consisted of the introduction of an experimental mouse of either genotype in the cage of a CD-1 aggressor until the first aggression occurred. Mice were then separated for 2 hrs by a transparent, perforated Plexiglas wall to allow visual and olfactory exposure. The separator was then removed, and the second attack occurred. Social defeat sessions were carried out once daily (on days 1-3, 5, 9) or twice daily (on days 6 and 10). The stress protocol included overcrowding sessions: 6/8 mice were placed together in a standard holding cage (33 × 15 × 13 cm) for 24 h (on days 4 and 8) with diet and water available ad libitum. Non-stressed mice were left undisturbed in their own home cages with other non-stressed mice (3-4 mice per cage). 24 hours after the last aggression all the c57bl/6 mice were tested in the social interaction test. Then different cohorts of animals were used for novel object recognition or novel object location tests. The experimental timeline is reported in Figure 17.

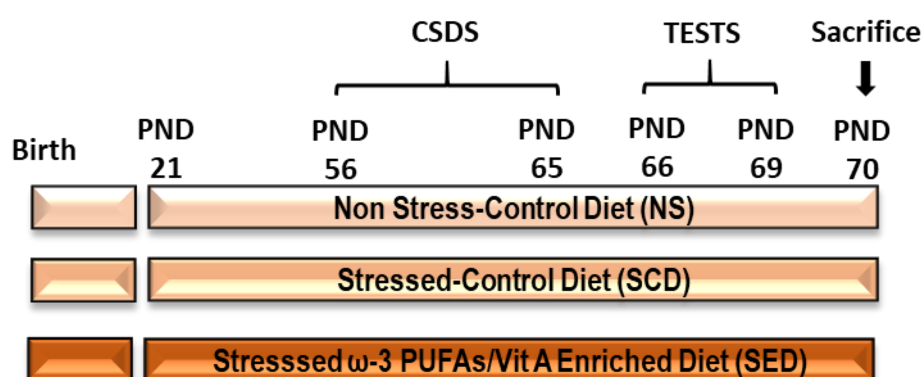


Figure 17. Time-line for the chronic social defeat stress experiment. Adult $HDC^{+/+}$ and $HDC^{-/-}$ mice were randomly assigned to three experimental groups: NS=Non-stressed, SCD=stressed fed with control diet, and SED=stressed fed with enriched diet

Social Interaction Test: Twenty-four hours after the last defeat session mice were subjected to the social interaction test adopting the protocol described by (Golden et al. 2011). Briefly, mice were habituated to an arena (41 cm x 32 cm x 40 cm) containing an empty wire-mesh enclosure (7.5 cm length, 9.5 cm width) and their movements recorded for 2.5 min to determine baseline exploratory behaviour and locomotion (T1). During the second session (T2) the wire-mesh contained an unfamiliar CD1 aggressive mouse and the time the experimental C57BL/6 mouse spent in its proximity was measured. Trials were video-recorded and analysed by an experienced observer unaware of the group assignment to time spent in the interaction zone, that is 5 cm around the wire mesh cage. Exploration was defined as sniffing or touching the cage with the nose and/or forepaws. Social interaction (SI) was calculated as the ratio between the time spent in the interaction zone during T2 and T1.

Novel object recognition and Novel object location test: The novel object recognition (NOR) and novel object location (NOL) tests were conducted to evaluate the short-term, non-spatial recognition and short-term spatial memory, respectively (Ennaceur and Delacour 1988). Both tests were conducted in a white polyvinylchloride rectangular chamber (70 × 60 cm and 30 cm high), with a grid floor that is easily cleaned, and illuminated by a 75-W lamp suspended 50 cm above the box. A video camera was positioned over the arena and used to record the animals' behaviour. The objects were gray polyvinyl chloride shapes: cubes of 8 cm side, pyramids and cylinders of 8 cm height. The object recognition task consisted of a training phase (T1) and a testing phase (T2). Twenty-four h prior to T1, mice were habituated for two 10 min-session to the experimental apparatus in the absence of any object.
In the NOR the mouse was placed for 5 min into the test arena facing the same direction and in the same position in the presence of two identical plastic objects (T1). T2 was performed 1 h after T1, during which, each mouse was again placed in the test arena for 5 min in the presence of one of the familiar objects and a novel object. The position of the objects (left/right) was randomized to prevent bias due to order or place preference. The behavior of mice during T2 was videotaped, and the exploration time of the familiar (F) and the new object (N) were measured.
NOL task was similar to that of NOR test. The only differences consisted in that during the test phase (T2) of NOL task, mice were re-exposed to the test area for 5 min, with one of the identical sample objects moved to a novel spatial location, and the other object remained in the original position. Placement of the moved (“novel”)/unmoved (“familiar”) or changed (“novel”)/unchanged (“familiar”) objects in the OLR and NOR tasks, respectively, followed a

counterbalanced design between mice to control for location effects. The objects and the floor of the arena were cleaned with a 30% ethanol solution between each phase to eliminate possible odours left by other animals. The behaviour of mice was videotaped, and the time spent actively exploring the objects was measured. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws.

Open field test: Mice locomotor activity and anxiety-like level were tested in an open arena (60 x 70 x 30 cm) where a virtual zone (20 x 23 cm) was delimited in the centre of the arena; mice were allowed to freely explore the arena for 10 minutes. In between observation, the arena was cleaned with 30 % ethyl alcohol in water to remove possible scent cues left by the animal. The time spent at the centre and periphery of the open field and total distance travelled were measured using a video tracking system and analysed using Smart 2.5 software.

Hippocampal slices preparation. As previously described (Mlinar et al. 2006, Mlinar et al. 2008, Morini et al. 2011) hippocampal slices were prepared from male mice (C57BL/6 strain) ~10 weeks old subsequently to social interaction test (SIT). Mice were anaesthetized with isoflurane and decapitated with a scissor cut. The hippocampi were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF), which contained the following (in mM): NaCl, 124; KCl, 2.75; NaH_2PO_4, 1.25; $NaHCO_3$, 26; $MgSO_4$, 1.3; $CaCl_2$, 2; D-glucose 10. The solution was bubbled with a 95% O2 ⁄ 5% CO2 gas mixture (pH 7.4). After discarding approximately 2 mm of the dorsal hippocampal pole, six transversal slices of 400 µm nominal thickness were cut with a McIlwain tissue chopper (Gomshall, UK) and kept for at least 1 h at room temperature until recording. Typically, two out of the six (dorsal–central) slices were used for LTP experiments.

Electrophysiological recordings. The slice was placed on a nylon mesh, completely submerged in a recording chamber and continuously superfused (1.9-2.0 mL / min) with oxygenated ACSF at 32–33 °C. The CA1 region was disconnected from the CA3 region by a surgical cut. Slices were incubated for 15 min in the recording chamber before initiating electrical stimulation that was continuous throughout the experiment. Synaptic responses of CA1 pyramidal neurons apical dendrites were elicited by stimulation of the Schaffer collateral / commissural pathway. Stimulation pulses (80 µs duration; 30 s interpulse interval), triggered by a PC controlled by WinLTP software (Anderson & Collingridge, 2001) were delivered by a stimulus isolation unit (DS2, Digitimer, Welwyn Garden City, UK) through a concentric bipolar

Pt-Ir electrode (125 µm / Rnd / 25µm). Field potentials were recorded with a glass electrode (filled with 150 mM NaCl, 2–10 MΩ resistance) placed in the distal third of the stratum radiatum to record field excitatory postsynaptic potentials (fEPSP). The distance between recording electrodes and the stimulating electrode was 300–400 µm (Figure 21A). Recorded potentials were amplified with Neurolog NL 104 amplifier (Digitimer), digitized with Digidata1320A (Axon Instruments) with the sampling rate of 5 kHz and stored in a PC for off-line analysis. At the beginning of each experiment, a stimulus–response curve (SRC), obtained by gradually increasing stimulus intensity, was recorded. The fEPSP was determined as the slope of the initial falling phase of the synaptic response recorded in the stratum radiatum. The stimulus intensity of test pulses was set to evoke a fEPSP that had an initial slope equal to 35–45% of the maximum obtained in the SRC. Unless otherwise stated, stimulus intensity was held constant throughout the remainder of the experiment. At least 20 min of stable responses were used to generate the baseline values, before inducing LTP.

LTP protocols. LTP was induced by theta-rhythm pattern consisting of a single train of 5 stimuli (100 Hz intraburst frequency with 5 Hz of burst frequency; TB5). Post-TB5 responses were followed for 1 h following which TB10 was delivered, following effect for 15 minutes. The response to a single TB10 was considered to represent the maximal potentiation achievable in a given slice, as delivery of further TB10 stimulations do not elicit significant increase in LTP (Morini et al., 2011).

Western blot analysis: For the neurochemical determination mice were sacrificed 24 hs after the end of novel object recognition test. After sacrifice, mice brains were dissected out on ice and cortices and hippocampi immediately isolated. The pooled structures (left and right) were individually homogenized in 400 µL ice-cold lysis buffer containing protease and phosphatase inhibitors (50mM TrisHCl (pH 7.5), 50mM NaCl, 10mM EGTA, 5mM EDTA, 2mM NaPP, 4mM PNFF, 1mM Na3VO4, 1.1mM PMSF, 20 µg/µL Leupeptin, 50 µg/µL Aprotinin, 0.1% SDS) and centrifuged at 12000 rpm at 4 °C for 15 minutes. The supernatant was collected and total protein levels were quantified using the Pierce BCA Protein Assay (Thermo Scientific, USA). Homogenates were diluted in a mix of lysis buffer and loading buffer 2x (50mM Tris pH = 6.8, 100mM DTT, 10% Glycerol, 1% Bromophenol Blue, and 2% SDS) and boiled for 10 minutes. Aliquots containing 15 µg of total proteins, for detection of synaptophysin, were resolved by electrophoresis on a 14% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon Transfer Membranes,

Millipore, USA). Blots were blocked in Tris-buffered saline, pH 7.6 containing 0.1% of Tween 20 (TBS-T) and 5% non-fat dry milk (Bio-Rad Laboratories, USA) for 2 h at room temperature and then incubated overnight at 4°C with antibodies against synaptophysin (1:10000 ThermoFisher Scientific) or tubulin (1:1000 Cell Signaling) all diluted TBS-T containing 5% non-fat dry milk. Immunodetection was performed with secondary antibodies (anti-rabbit IgG conjugated to horseradish peroxidase, Cell Signaling technology, USA) diluted 1:5000 in TBS-T containing 1% of non-fat dry milk. Membranes were washed in TBS-T and then reactive bands were detected using enhanced chemiluminescence (Luminata Crescendo, Millipore, USA). Quantitative densitometric analysis was performed using the QuantityOne analysis software (Bio-Rad). For each sample, a ratio of Synaptophysin/Tubulin densities was calculated and then all the individual rates were expressed as a percentage of the average of ratios obtained from control groups ($HDC^{+/+}$ Non stressed).

Real-Time PCR of gene expression in the hippocampus and prefrontal cortex: The hippocampus and prefrontal cortex were rapidly removed and stored at –80°C (see Figure 17 for the timeline). RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies™, Saint-Aubin, France). RNA concentrations were determined using a Nanodrop ND-1000 (Labtech). Using OligodT and random primers (Invitrogen), cDNA was synthesized with SuperScript IV Reverse Transcriptase (Invitrogen, Life Technologies™, Saint-Aubin, France). Briefly, 1 µg of total RNA mixed with RNasin (Invitrogen, Life Technologies™, Saint-Aubin, France) and DNase (Invitrogen, Life Technologies™, Saint-Aubin, France) was incubated at 37°C. Then, OligodT and random primers were added for incubation at 65°C. Then, the SuperScript IV mix was added, and the mixtures were incubated at 23°C for 10 min, followed by 50°C for 10 min and 80°C for 10 min.

To measure retinoic acid receptors expression, quantitative PCR 217 was performed using SYBR® assay (Eurogentec, Seraing, Belgium). Real-time PCR was performed using the LightCycler 480 system with a ninety-six-well format (Roche Diagnostics) in a final volume of 10 µl, containing 1×LightCycler 480 SYBR Green I Master solution, 0.5 µM of each primer and 7 µl of cDNA. The following program started with an initial denaturation step for 10 min at 95°C, then an amplification for 45 cycles (10 s denaturation at 95°C, 6 s annealing at 62°C, and 10 s extension at 72°C), finally a melting curve analysis was run.

The forward- and reverse-primer sequences and the amplicon size for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *RAR-α and RXR-α* are summarised in Table

4. *GAPDH* was used as the reference gene, since its expression level was unaffected under our experimental conditions. Quantification data were analysed using LightCycler 480 Relative Quantification software (version 1.5).

Table 4. Primers used for LightCycler RT-qPCR

Gene name	Nucleotide sequence 5'-3'	Product length (bp)
GAPDH	F: CCAGTGAGCTTCCCGTTCA R: GAACATCATCCCTGCATCCA	78
RAR-α	F: GGCGAACTCCACAGTCTTAATG R: GCTGGGCAAGTACACTACGAAC	118
RXR-α	F: GATTCCGATACGACGACAGT R: CATCACCACTCTCGCCATC	141

To measure fatty acid metabolic enzymes and RXR-β, quantitative PCR was performed using the Applied Biosystems (Foster, CA) assay-on demand gene expression protocol as previously described (Mingam et al., 2008). Briefly, cDNAs for (5-LOX, 12-LOX, COX2, CYP1A1, soluble hydrolase (she), RXR-β and a housekeeping gene (GAPDH) will be amplified by PCR using an oligonucleotide probe with a 5′ fluorescent reporter dye (6-FAM) and a 3′ quencher dye (NFQ). PCR program consisted of 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence will be measured using an AB 7500 Real-Time PCR system (Applied Biosystems, Foster city, CA). The data of real time PCR are expressed as Relative Quantification.

Statistical analysis: Data from behavioural and neurochemical experiments were analysed using Graphpad Software (version 6.0). The data presented in graphs or tables are expressed as mean and S.E.M. or mean ± S.D., respectively. Statistical significance was determined using Two-way ANOVA followed by Bonferroni's multiple comparison post-hoc tests. The level of significance was set to $p<0.05$. Data from electrophysiology experiments were analysed using WinLTP and Prism 6 software (GraphPad Software, San Diego, CA, USA). For statistical comparison of changes in LTP experiments, the steady-state values were computed by averaging 21 consecutive responses obtained over 5 min period immediately before the theta burst stimulation (baseline value) and at 55-60 min after TB5. Typically, more than one slice was used per mouse and the results of all determinations per genotype and treatment are shown and analysed in Fig. X to account for the overall variability of LTP responses in the different genotypes. Mean values from replicates in the same animal were used for genotype vs condition statistical analysis reported in results. Unless otherwise stated, numbers represent experiments carried out in slices taken from different animals.

Results

ω-3 PUFA and Vitamin A enriched diet prevented body weight reduction induced by chronic stress in HDC$^{+/+}$ mice

Figure 18 shows the effect of ω-3 PUFA and Vitamin A enriched diet in mice submitted to the chronic social defeat stress on body weight and food consumption. Stressed mice fed control diet (SCD) of both genotypes gained less body weight compared to non-stressed controls (NS). The supplemented diet prevented this body weight reduction in HDC$^{+/+}$, but not in HDC$^{-/-}$ mice (Two-way ANOVA and Bonferroni MCT $F_{(Interaction)2,\ 179}$=4,804; $F_{(Diet)2,\ 179}$=14,4; $F_{(Genotype)1,\ 179}$=3,511) (Figure 18A). Figure 18B show the cumulative food consumption expressed in grams per body weight in HDC$^{+/+}$ and HDC$^{-/-}$ mice fed with enriched or control diet and subjected to the CSDS protocol Two-way ANOVA revealed no differences between groups (Two-way ANOVA and Bonferroni MCT $F_{(Interaction)2,\ 179}$=2,73; $F_{(Diet)2,\ 179}$=1,250; $F_{(Genotype)1,\ 179}$=0,0003412).

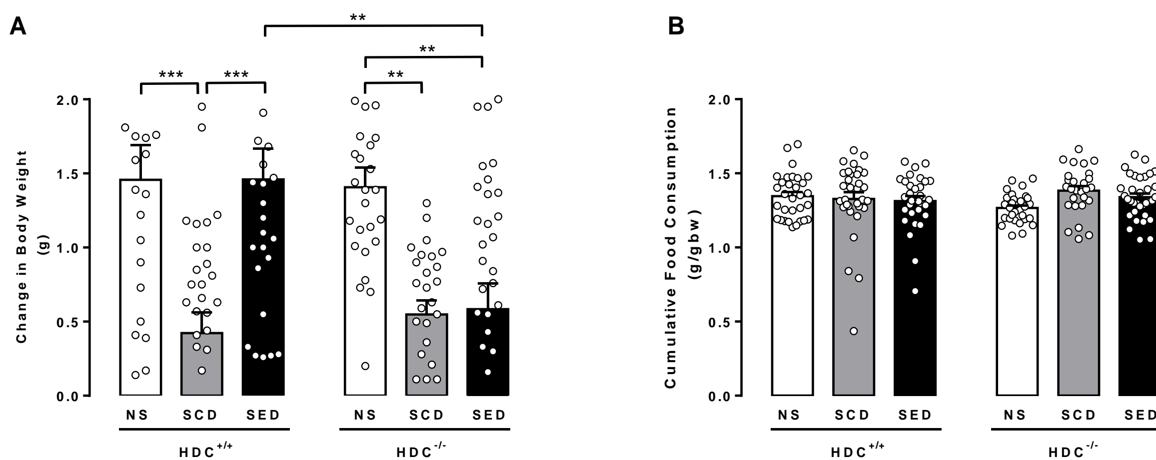
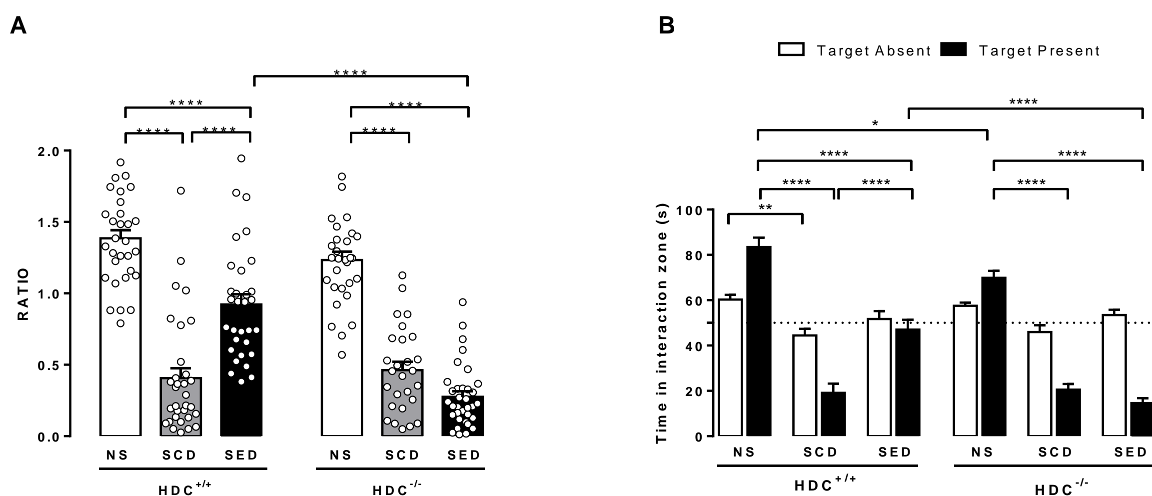


Figure 18. **Effects of chronic social defeat stress on body weight and food consumption. (A) CSDS induced reduced body weight gain in HDC$^{+/+}$ and HDC$^{-/-}$ fed with control diet compared with non-stressed control groups; the enriched diet prevented this reduction only in HDC$^{+/+}$ mice. (B) Cumulative food consumption of the different experimental groups. (Two-way ANOVA and Bonferroni MCT; ***p<0.001, **p<0.01, n=27-34).**

ω-3 PUFA and Vitamin A enriched diet reduces social avoidance induced by CSDS

Figure 19 shows the effect of ω-3 PUFA and Vitamin A enriched diet in SED mice compared to SCD and NS animals. The results are expressed both as ratio (Figure 19A) and as absolute times (sec) of exploration in the absence or presence of the social stimulus (Figure 19B). The time spent in the interaction with the aggressive mouse was influenced by stress exposure. Defeated mice of both genotypes fed with control diet spent significantly less time in social interaction compared to NS mice (****$p<0.0001$). SED $HDC^{+/+}$ mice though, spent significantly more time in the interaction zone compared to SCD mice (****$p<0.0001$). The surprising result is that the effect of the diet is lost in SED $HDC^{-/-}$ animals suggesting that the central histaminergic system is essential for the effects of the enriched diet (****$p<0.0001$) (Two-way ANOVA and Bonferroni MCT $F_{(Interaction)2,\ 179}$=18,21; $F_{(Diet)2,\ 179}$=117,9; $F_{(Genotype)1,\ 179}$=25,3).

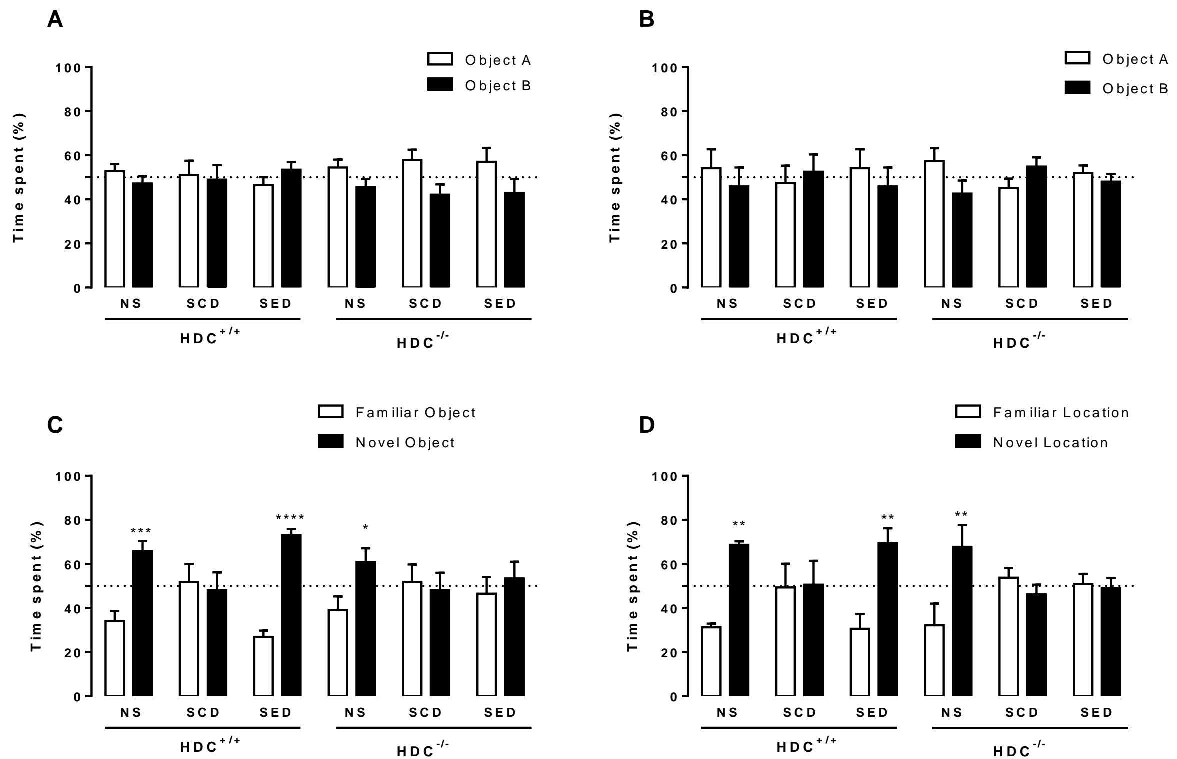
Figure 19B shows the same results expressed as seconds spent in the interaction zone in absent (white bar) or presence (black bar) of the social target. Again, we observed that stressed mice spent less time exploring the aggressive mouse compared to non-stressed controls (****$p<0.0001$) and $HDC^{+/+}$ stressed mice fed with enriched spent more time exploring the aggressive CD1 compared to stressed mice fed with control diet (****$p<0.0001$). Also, SED, $HDC^{-/-}$ animals failed to respond to the beneficial effects of the supplemented diet(****$p<0.0001$; Two-way ANOVA and Bonferroni MCT $F_{(Interaction)5,358}$=31,33; $F_{(Group)5,358}$=62,39; $F_{(Target)1,358}$=29,87).



<u>Figure 19.</u> Social defeat stress induced social avoidance in $HDC^{+/+}$ and $HDC^{-/-}$ mice. (A) Social interaction time is expressed as ratio between the time spent near the cage with and without the stimulus mouse. In $HDC^{-/-}$ ω-3 PUFA/Vitamin A enriched diet did not increase interaction time compared to controls. (B) Social interaction results expressed as absolute time spent in the interaction zone in absence (white bar) or presence (black bar) of social stimulus (Two-way ANOVA and Bonferroni MCT: *$p<0.05$; **$p<0.01$; ****$p<0.0001$; n=27-34).

ω-3 PUFA and Vitamin A enriched diet ameliorates the memory impairment induced by CSDS in $HDC^{+/+}$ mice but not in $HDC^{-/-}$

Figure 20 shows the effect of stress and of ω-3 PUFA/Vit A enriched diet on mice performances in the novel object recognition (NOR) and novel object location (NOL) tests. No significant group effects were detected during T1 of both tests (data not shown). In the NOR, during T2 performed 1 hour after the T1, both NS $HDC^{+/+}$ and NS $HDC^{-/-}$ mice spent more time exploring the familiar object (Two-way ANOVA and Bonferroni MCT $F_{(Interaction)5,70}$=5,30; $F_{(Groups)5,70}$=7,387e^{-14}; $F_{(Object)1,70}$=20,97). Stressed mice of both genotypes did not discriminate between the familiar and novel object; however, $HDC^{+/+}$ stressed mice fed with ω-3 PUFA and Vitamin A enriched diet spent significantly more time exploring the new object rather than the familiar one (**** $p<0.0001$). On the contrary, the enriched diet had no effect on the discrimination of $HDC^{-/-}$ stressed mice (Figure 20A). When we tested the spatial memory in NOL, we observed the same results obtained in NOR. Indeed, NS mice of both genotypes spent more time exploring the object placed in a new location compared to the one placed in the familiar zone (**$p<0.01$). SCD mice of both genotypes showed memory impairment because they did not recognize the object placed in the familiar location and they spent equal time exploring the novel and the familiar location (Two-way ANOVA and Bonferroni MCT $F_{(Interaction)5,58}$=4,797; $F_{(Groups)5,58}$=1,476e^{-14}; $F_{(Object)1,58}$=17,58). In this test as well, SED $HDC^{+/+}$ mice spent more time exploring the object placed in the new location than the familiar one (**$p<0.01$) indicating a role of brain histamine in the effect of the diet in preventing recognition and spatial memory impairment induced by stress (Figure 20B).



<u>Figure 20.</u> Effect of ω-3 PUFA/Vitamin A enriched diet on mice performances in the novel object recognition and novel object location test. A-B Percentage of time spent exploring the two identical object A and B during training. C-D Percentage of time spent exploring the familiar (black columns) and novel (white columns) objects. (C) Performance of animals in NOR (D)Performance of mice in NOL. (Two-way ANOVA and Bonferroni's MCT: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; n=5-9).

CSDS does not affect motility of mice tested in the open field

When evaluated in the open field test, two-way ANOVA revealed no significant differences between experimental groups independently of diet, stress and genotype in term of distance travelled and time spent moving (Table 5). Neither stress nor diet affected the number of entries and time spent in the centre or the periphery of the open field indicating that CSDS did not induce anxiety-like behaviours (Table 5).

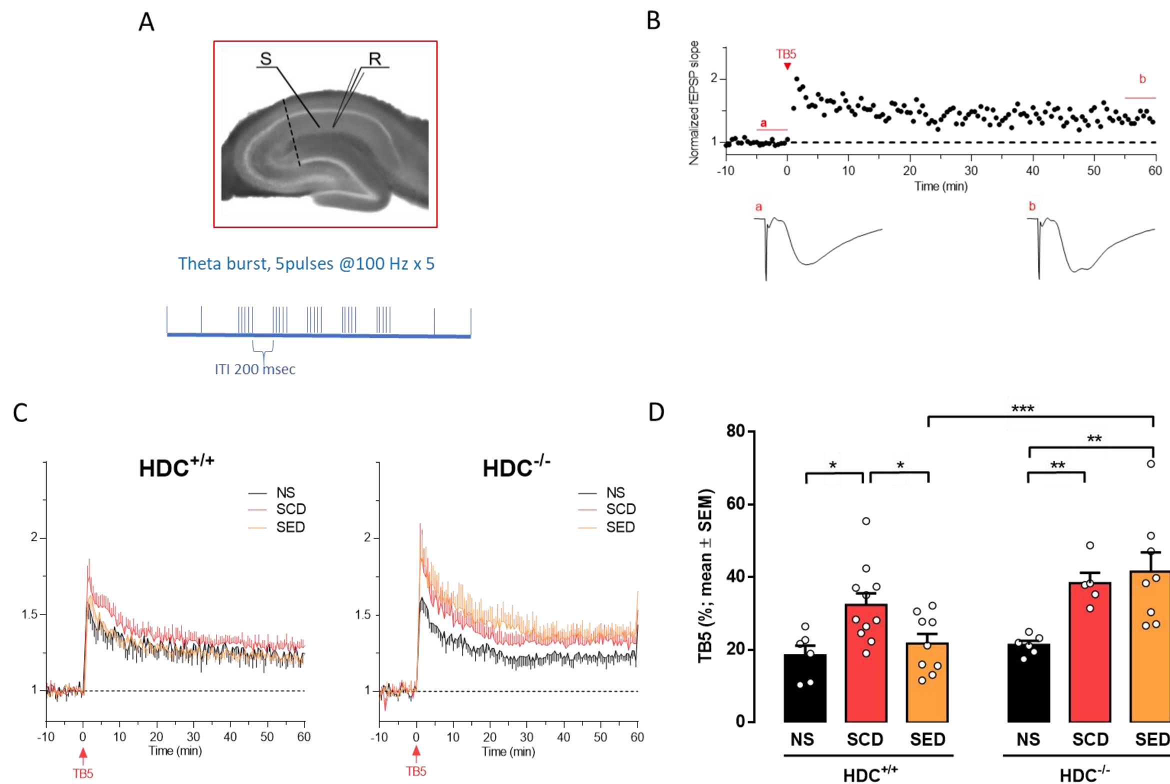
<u>Table 5.</u> CSDS did not alter locomotor activity and did not induce anxiety-like behaviour when measured in the open field arena. Results are expressed ad means ± SD of 5-9 animals per experimental group. F ratio and P values were determined using Two-way ANOVA

		$HDC^{+/+}$			$HDC^{-/-}$				
Parameter	**Zone**	**NS**	**SCD**	**SED**	**NS**	**SCD**	**SED**	**F ratios**	**P value**
Entries	Central	16,6 ±4,7	15,33 ± 5,76	16,92 ± 5,14	16,33 ± 4,76	17,08 ± 6,54	15,87 ± 4,90	Interaction F (2, 76) = 0,4731 Genotypes F (1, 76) = 0,01577 Conditions F (2, 76) = 0,01667	0,625 0,9 0,983
Time spent (s)	Central	42,91 ± 17,90	38,51 ± 10,79	44,27 ± 18,09	47,5 ±17,35	48,84 ± 27,66	43,07 ± 11,13	Interaction F (2, 76) = 0,6784 Genotypes F (1, 76) = 1,348 Conditions F (2, 76) = 0,07071	0,51 0,25 0,931
	Peripheral	544,8 ± 27,91	561,42 ± 10,97	555,43 ± 18,05	552,1 ± 17,35	550,7584 ± 27,66	556,525 ± 11,13	Interaction F (2, 76) = 1,367 Genotypes F (1, 76) = 0,02911 Conditions F (2, 76) = 1,366	0,261 0,865 0,261
Distance travelled (cm)	Central	487,62 ± 116,21	467,26 ± 213,59	500,39 ± 160,07	520,11 ± 244,14	469,6246 ± 139,60	459,6144 ± 140,19	Interaction F (2, 76) = 0,3171 Genotypes F (1, 76) = 0,002588 Conditions F (2, 76) = 0,2941	0,729 0,96 0,74
	Peripheral	4916,34 ± 825,80	5336,63 ± 798,84	5000,23 ± 1157,67	5020,093 ± 741,39	4974,674 ± 1005,60	4915,333 ± 692,65	Interaction F (2, 76) = 0,4802 Genotypes F (1, 76) = 0,3501 Conditions F (2, 76) = 0,4165	0,62 0,556 0,66
	Total	5403,97 ± 848,62	5776,78 ± 886,75	5456,97 ± 1288,74	5540,20 ± 755,54	5444,298 ± 961,45	5374,947 ± 699,33	Interaction F (2, 76) = 0,4455 Genotypes F (1, 76) = 0,2112 Conditions F (2, 76) = 0,3085	642 0,647 0,735
Time spent Moving (s)	Central	24,43 ± 12,03	15,42 ± 6,52	21,1 ± 11,41	20,06 ± 9,39	17,55 ± 7,91	16,26 ± 5,56	Interaction F (2, 76) = 1,158 Genotypes F (1, 76) = 1,353 Conditions F (2, 76) = 2,776	0,32 0,248 0,069
	Peripheral	197,13 ± 56,58	188,47 ± 90,10	159,93 ± 61,94	198,66 ± 42,37	165,67 ± 38,60	200,15 ± 39,60	Interaction F (2, 76) = 2,065 Genotypes F (1, 76) = 0,2534 Conditions F (2, 76) = 1,122	0,134 0,616 0,331
	Total	221,57 ± 62,84	203,88 ± 89,84	181,03 ± 71,26	218,72 ± 43,13	183,22 ± 42,21	216,41 ± 40,30	Interaction F (2, 76) = 1,512 Genotypes F (1, 76) = 0,08892 Conditions F (2, 76) = 1,563	0,228 0,767 0,216

ω-3PUFAs and vitamin A enriched diet prevents the stress-induced changes in synaptic plasticity of apical dendrites in hippocampus only in $HDC^{+/+}$ mice

The hippocampus is one of the brain structures most involved in the regulation of stress responses (McEwen 2016). To learn the effect of stress and the enriched diet on synaptic plasticity in the two genotypes, we assessed the magnitude of LTP in hippocampal brain slices. Hippocampal LTP was evoked by one brief train of electrical stimulation, TB5, that mimics the physiological theta rhythm and leads to a sustained increase in synaptic transmission efficacy. TB5 produced a significant increase of fEPSP slope with respect to baseline in all groups (data not shown). Slices from both $HDC^{+/+}$ SCD and $HDC^{-/-}$ SCD mice responded to TB5 stimulation with a larger LTP compared to that of non-stressed mice of the corresponding genotype (Figure 21). Two-way ANOVA revealed a significant genotype x condition interaction ($F_{(Interaction)2.57}$=3.263; $F_{(Genotype)1.57}$=11.31; $F_{(Conditions)2.57}$=8.391); Figure 21D). Post hoc analysis confirmed increased LTP in stressed mice of $HDC^{+/+}$ ($p < 0.05$) and $HDC^{-/-}$ ($p < 0.01$) mice

relative to non-stressed controls. The enriched diet prevented the increased LTP magnitude of $HDC^{+/+}$ SED mice, but was ineffective in $HDC^{-/-}$ SED mice ($p<0.001$; Figures 21D) indicating that the presence of histamine *in vivo* is required for the recovery of synaptic plasticity to normal levels promoted by the diet.



<u>Figure 21.</u> Histamine deficiency prevents ω3-PUFA and Vitamin A enriched diet modulation of synaptic plasticity in dorsal hippocampal apical dendrites. (A) Positioning of stimulation electrode (S) on Schaffer collaterals and recording electrode (R) in CA1. The dashed line indicates a cut made between CA1 and CA3 to prevent recurrent propagation of action potentials. (B) top panel, time-course of a representative experiment indicating the steady-state values used for fEPSP slope calculation (a, b); lower traces, representative steady-state averaged traces in baseline (a) and 55-60 min after TB5 (b). (C) Averaged time-courses of responses before and after delivery of one TB5 (arrow) in slices obtained from $HDC^{+/+}$ (left panel) and $HDC^{-/-}$ (right panel) mice (NS, SCD, SED $HDC^{+/+}$ mice; n=6,11,9; NS, SCD, SED $HDC^{-/-}$ mice; n=6,5,8). (D) Histograms report the LTP produced by TB5 stimulation in $HDC^{+/+}$ (left) and $HDC^{-/-}$ (right) mice. Data in columns are expressed as mean ± SEM of the percent increase in responses measured 55-60 min after LTP induction with respect to baseline as in (C). (TB5: n=8,11,9 $HDC^{+/+}$ and n=6,5,7 $HDC^{-/-}$; two-way ANOVA, and Bonferroni MCT; *p<0.05, **p<0.01, ***p<0.0001).

ω-3 PUFA and Vitamin A enriched diet increases synaptophysin levels in the hippocampus of $HDC^{+/+}$ mice

Figure 22 shows the effects of stress and diet supplementation on synaptophysin level in the hippocampus (Figure 22A) and prefrontal cortex (Figure 22B). In the hippocampus, two-way ANOVA showed significant differences among experimental groups and significant interaction between variables ($F_{(Interaction)2,35}$=6,121; $F_{(Genotype)1,35}$=0,1190; $F_{(Conditions)2,35}$=1,203). Bonferroni's post hoc test showed that ω-3 PUFA and Vitamin A enriched diet significantly increased synaptophysin levels in the hippocampus of $HDC^{+/+}$ mice compared with SCD or NS mice. However, ω-3 PUFA and Vitamin A enriched diet was ineffective in $HDC^{-/-}$ mice as synaptophysin expression was not different from that of $HDC^{-/-}$ stressed mice fed with control diet or non-stressed mice (Figure 22A).

In the prefrontal cortex, two-way ANOVA revealed no differences regarding the stress, diet or genotype (Two-way ANOVA and Bonferroni MCT $F_{(Interaction)2,35}$=2,748; $F_{(Genotype)1,35}$=0,9513; $F_{(Conditions)2,35}$=1,476) (Figure 22B).

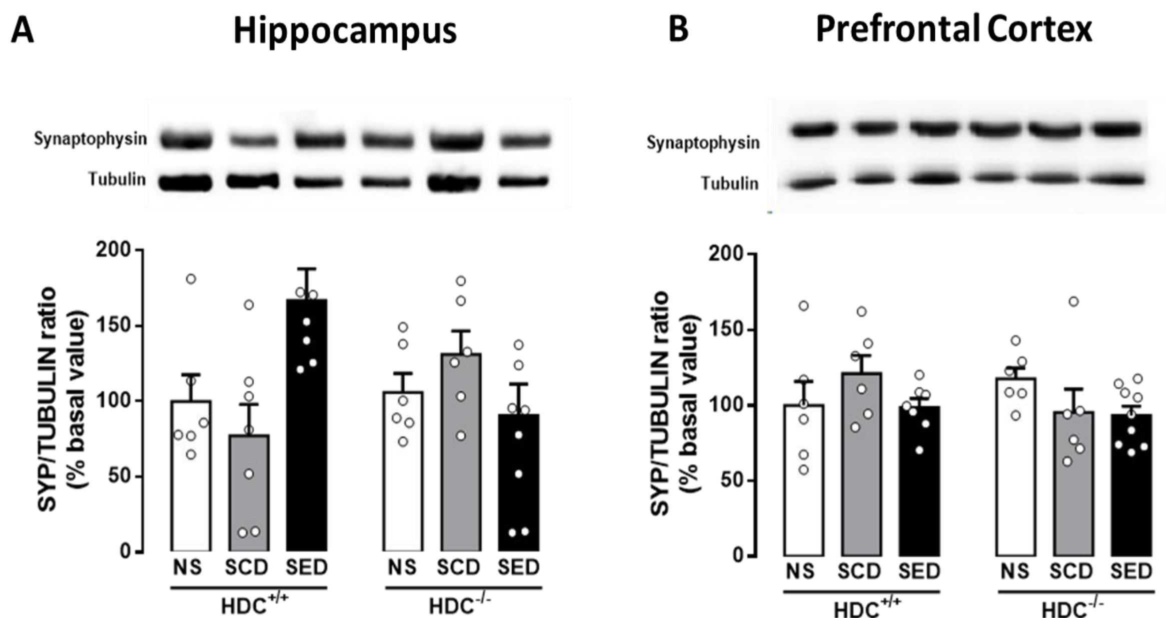


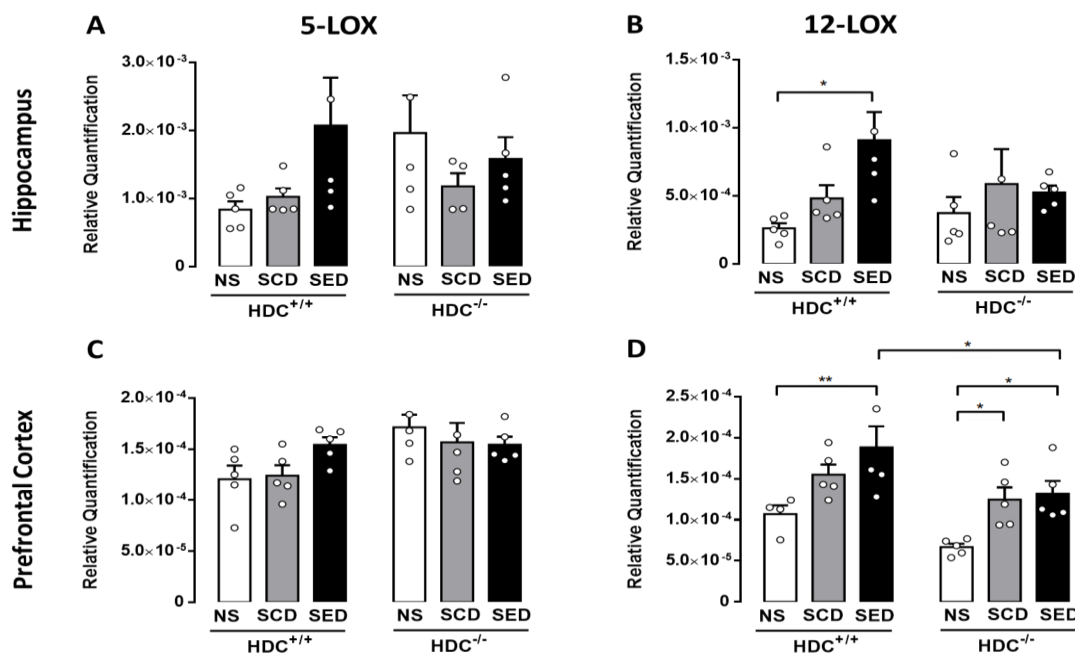
Figure 22. **Effect of an ω-3 PUFA/Vitamin A enriched diet on synaptophysin levels of mice hippocampus (A) and prefrontal cortex (B). Enriched diet increased Synaptophysin levels in hippocampal homogenates from normal ($HDC^{+/+}$) but not from histamine depleted mice ($HDC^{-/-}$) subjected to 10 days-chronic social defeat stress (Two-way ANOVA and Bonferroni MCT; n=6-9)**

Effects of ω3-PUFA and Vitamin A supplemented diet on hippocampal and cortical gene expression of enzymes of fatty acid metabolism

To study the fatty acid metabolic pathways, we examined whether the enriched diet modulated the hippocampal and cortical expression of some metabolic enzymes, particularly focusing on lipoxygenases, such as 5-LOX and 12-LOX.

Two-way ANOVA revealed no effect of stress or diet on the hippocampal mRNA expression of *5-LOX* (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,23}$=1,988; $F_{(Genotypes)1,23}$=0,5971; $F_{(Conditions)\ 2,23}$=1,519) (Figure 23A); indeed, an increase in mRNA *12-LOX* levels was observed in $HDC^{+/+}$ stressed mice fed the with enriched diet, but not in hippocampus of SED $HDC^{-/-}$ mice (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}$=1,785 p=0,1894; $F_{(Genotypes)1,24}$=0,1961 p=0,6618; $F_{(Conditions)\ 2,24}$=3.537 $p<0.05$) (Figure 23B).

In the prefrontal cortex, two-way ANOVA showed a genotype effect on 5-LOX (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}$=2.157 p=0,1376; $F_{(Genotypes)1,24}$=7.67 $p<0.05$; $F_{(Conditions)\ 2,24}$=0,6399 p=0.5361; Figure 23C). We observed effects of genotype and conditions in 12-LOX expression (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,23}$=0.3713 p=0.6939; $F_{(Genotypes)1,23}$=10.88 $p<0.01$; $F_{(Conditions)\ 2,23}$=11.22 $p<0.001$; Figure 23D) in the prefrontal cortex of SED $HDC^{+/+}$ compared to NS $HDC^{+/+}$ mice. Stress increased 12-LOX expression in the cortex of $HDC^{-/-}$ mice, but the diet had no effects.



<u>Figure 23</u>. Effect of enriched diet on hippocampal and cortical fatty acid metabolic enzymes. Supplemented diet increased 12-LOX expression in the hippocampus of $HDC^{+/+}$ mice and 12-LOX expression in $HDC^{+/+}$ and $HDC^{-/-}$ animals but the increase in $HDC^{+/+}$ is greater in normal mice compared to histamine-depleted ones. (A-C) 5-LOX, (B-D)12-LOX mRNA expression measured by RT-qPCR. Data are represented as Relative Quantification vs GAPDH. (Two-way ANOVA and Bonferroni's MCT; *p<0.05; ** p<0.01 n=4-5).

Then we analysed the expression of other enzymes involved in the metabolism of fatty acids including COX-2, CYP1A1 and EPHX2. Two-way ANOVA revealed no statistical differences between the groups and the results are shown in Table 6

Table 6. Expression of some enzyme involved in fatty acid metabolism. COX-2= Cyclooxygenase 2; CYP1A1= Cytochrome 1A1; sEH= soluble epoxide hydolases. Results are expressed as means ± SD of 4-5 animals per experimental group. F ratios and P values were determined using Two-way ANOVA.

	$HDC^{+/+}$			$HDC^{-/-}$				
Enzyme	NS	SCD	SED	NS	SCD	SED	F ratios	P value
COX2	18,74 ± 4,067	21,52 ± 6,71	35,2 ± 28,28	30,00 ± 21,86	17,02 ± 6,81	19,12 ± 2,91	Interaction F(2,22) = 1,776 Genotypes F(1,22) = 0,2656 Conditions F(2,22) = 0,5681	P =0,1927 P =0,6114 P =0,5747
CYP1A1	0,000186 ± 0,000070	0,0002866 ± 0,00013	0,00023175 ± 0,000056	0,0002204 ± 0,00012	0,00018525 ± 0,000063	0,00016125 ± 0,000035	Interaction F(2,21) = 1,477 Genotypes F(1,21) = 1,719 Conditions F(2,21) = 0,4782	P =0,2511 P =0,2039 P =0,6265
sEH	0,009308 ± 0,0018	0,010694 ± 0,0024	0,01235 ± 0,0019	0,017642 ± 0,011	0,0094325 ± 0,0019	0,012038 ± 0,0050	Interaction F(2,22) = 2,306 Genotypes F(1,22) = 1,209 Conditions F(2,22) = 0,9529	P =0,1233 P =0,2834 P =0,4010
COX2	5,63 ± 1,43	5,48 ± 1,91	5,45 ± 1,47	3,81 ± 0,44	4,75 ± 1,74	4,04 ± 0,58	Interaction F(2,24) = 0,4020 Genotypes F(1,24) = 6,874 Conditions F(2,24) = 0,2492	P =0,6734 P =0,0149 P =0,7815
CYP1A1	0,00003254 ± 0,000016	0,00003714 ± 0,000010	0,00003314 ± 0,000011	0,0000199 ± 0,0000056	0,00003046 ± 0,0000084	0,00002782 ± 0,000010	Interaction F(2,24) = 0,3401 Genotypes F(1,24) = 4,541 Conditions F(2,24) = 1,296	P =0,7151 P =0,0435 P =0,2922
sEH	0,002542 ± 0,00048	0,002102 ± 0,00071	0,002192 ± 0,00049	0,00181 ± 0,00017	0,001992 ± 0,00044	0,001756 ± 0,00037	Interaction F(2,24) = 1,084 Genotypes F(1,24) = 6,097 Conditions F(2,24) = 0,4687	P =0,3542 P =0,0210 P =0,6314

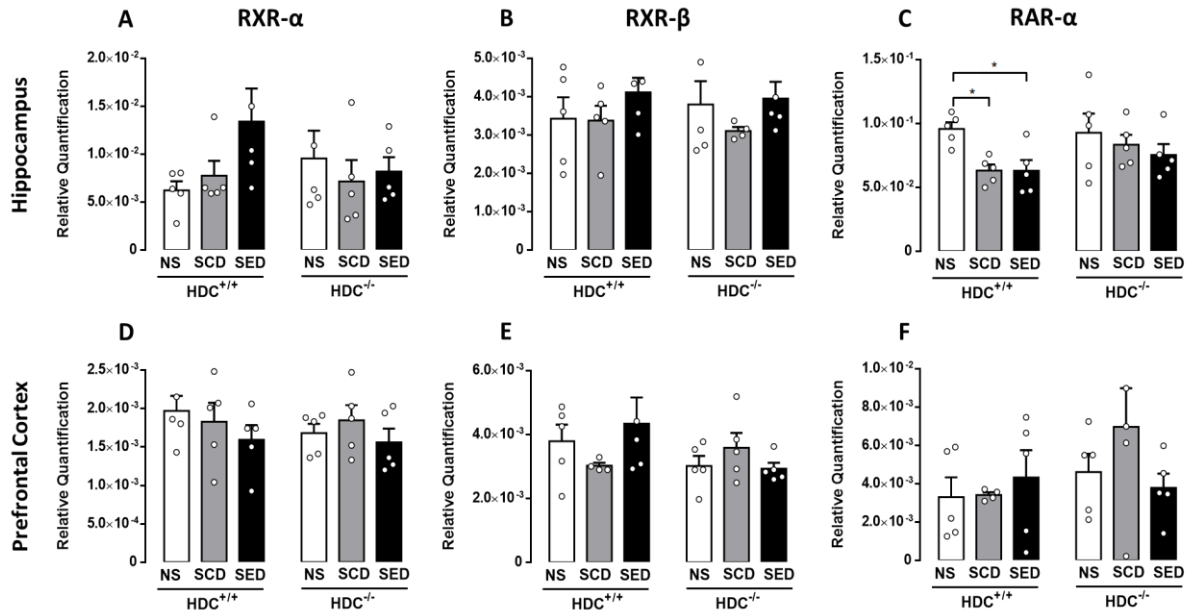
Effects of ω3-PUFA and Vitamin A supplemented diet on hippocampal and cortical gene expression of Retinoic acid receptors: RAR-α, RXR-α, RXR-β

To study the effect of vitamin A supplementation on stress responses, we examined whether the enriched diet modulated the hippocampal and prefrontal cortex expression of some retinoic acid receptors, such as RAR-α, RXR-α and RXR-β.

Two-way ANOVA analysis showed no differences on hippocampal RXR-α (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,23}$=0.2759; $F_{(Genotypes)1,23}$=0.002757; $F_{(Conditions)\ 2,23}$=1.447) and RXR-β expression (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}$=1.794; $F_{(Genotypes)1,24}$=0.2024; $F_{(Conditions)\ 2,24}$=1.293; Figure 24A-B). On the contrary a statistically significant difference was found in RAR-α expression in the hippocampus (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2,24}$=0,6012; $F_{(Genotypes)1,24}$=0,1648; $F_{(Conditions)\ 2,24}$=3,028) (Figure 24C) of $HDC^{+/+}$ stressed mice compared to NS group, but no differences were observed in $HDC^{-/-}$ mice, underlining, also in this case, a difference in the gene expression between $HDC^{+/+}$ and $HDC^{-/-}$.

In the prefrontal cortex Two-way ANOVA revealed no differences in RXR-α (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2.23}$=2.099; $F_{(Genotypes)1.23}$=1.889; $F_{(Conditions)\ 2.23}$=0.2463), RXR-β α (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2.24}$=0.3707;

$F_{(Genotypes)1.24}$=0.4218; $F_{(Conditions)\ 2.24}$=1.58) and RAR-α expression (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)2.23}$=1.324; $F_{(Genotypes)1.23}$=1.979; $F_{(Conditions)\ 2.23}$=0.5793) (Figure 24D-F).



<u>Figure 24.</u> Effect of enriched diet on hippocampal Retinoic acid receptors. (A-D) RXR-α, (B-E) RXR-β, (C-F) RAR-α mRNA expression measured by RT-qPCR. Data are represented as Relative Quantification vs GAPDH. (Two-way ANOVA and Bonferroni's MCT; *P<0.05; n=4-5).

Part IIb: Brain histamine and Oleoylethanolamide restore behavioural deficits induced by chronic social stress in mice.

Histamine and oleoylethanolamide (OEA) are phylogenetically old molecules that have been described in several species, (Panula and Nuutinen 2013, Piomelli 2013). In our laboratory it was recently demonstrated that all central actions of OEA that we investigated, invariably necessitate the activation of the brain histaminergic system (Provensi et al. 2014, Provensi et al. 2017, Costa et al. 2018). Here, we explored the relationship between the histaminergic system and OEA on the behavioural outcomes of chronic social defeat stress (CSDS), a preclinical paradigm that more closely reproduces some of the symptoms observed in depression (Menard et al. 2017). Exposure to frequent stressful stimuli as in the case of social stress, may cause maladaptive emotional reactions that increase the risk of depression, anxiety and cognitive impairments.

The manuscript Rani et al., has been submitted to Neurobiology of Stress.

Materials and Methods

Animals: Histidine decarboxylase null (C57bl/6, $HDC^{-/-}$) and wild type (C57bl/6, $HDC^{+/+}$) mice were grown in the Centro Stabulazione Animali di Laboratorio (CeSAL), Università di Firenze in humidity, temperature (22 - 24 °C) and light (light on 7:00-19:00)-controlled room. Mice were allowed free access to food and water. At postnatal (PND) day 21, mice were weaned and fed with standard chow diet (Mucedola s.r.l., Milan, Italy). Nine to 13-week-old male CD1 retired breeders (Charles River, Italy) were screened for aggressive behaviour and used for the social defeat stress protocol according to (Golden et al. 2011). All experiments were performed in accordance with the EEC recommendations for the care and use of laboratory animals (2010/63/EU) and approved by the Animal Care Committee of the University of Florence and Italian Ministry of Health (authorization n. 114-2017PR) and complying to the 3R. Ethical policy of the Università di Firenze complies with the Guide for the Care and Use of Laboratory Animals of the Council Directive of the European Community (2010/63/EU) and the Italian Decreto Legislativo 26 (13/03/2014). Every effort was made to minimize animal suffering and to reduce the number of animals used. All animals were weighted, and food consumption calculated daily. OEA (Tocris Bioscience, UK) was dissolved in saline/polyethylene glycol/ Tween80 (90/5/5, v/v). OEA (10mg/kg) or vehicle treatments started 10 days before the end of stress procedure.

Chronic Social Defeat Stress: C57bl/6 mice were singly housed prior to undergoing social defeat stress. CD1 mice were used as resident aggressors for the social defeat stress and were singly-housed prior to the experiments. Aggressive CD-1 mice, as defined by demonstrating at least one successful act of aggression during two consecutive days toward another male CD1 intruder mouse, were selected for use in the social defeat. A group of $HDC^{+/+}$ and of $HDC^{-/-}$ mice that received vehicle and a group of mice of either genotype that received OEA were subjected to the CSDS protocol for 21 days (PND56 to PND76); adapted from (Golden et al. 2011). Briefly, the procedure consisted of the introduction of an experimental mouse of either genotype in the cage of a CD-1 aggressor until the first aggression occurred. Mice were then separated for 2 hrs by a transparent, perforated Plexiglas wall to allow visual and olfactory exposure. The separator was then removed, and the second attack occurred. Social defeat sessions were carried out once daily (on days 1-4, 7-10, 15, 16, 19-21) or twice daily (on days 6, 12 and 17). The stress protocol included overcrowding sessions: 6/8 mice were placed together in a standard holding cage (33 × 15 × 13 cm) for 24 h (on days 5-6, 11-12, 18-19) or 48 h (on days 13–15) with diet and water available ad libitum. Non-stressed mice were left undisturbed in their own home cages with other non-stressed mice (4 mice per cage).

Open field test: Mice locomotor activity and anxiety-like level were tested in an open arena (60 x 70 x 30 cm) where a virtual zone (20 x 23 cm) was delimited in the centre of the arena; mice were allowed to freely explore the arena for 10 minutes. In between observation, the arena was cleaned with 30 % ethyl alcohol in water to remove possible scent cues left by the animal. The time spent at the centre and periphery of the open field and total distance travelled were measured using a video tracking system and analysed using Smart 2.5 software.

Social Interaction Test: Twenty-four hours after the last defeat session mice were subjected to the social interaction test adopting the protocol by (Golden et al. 2011). Briefly, mice were habituated to an arena (41 cm x 32 cm x 40 cm) containing an empty wire-mesh enclosure (7.5 cm length, 9.5 cm width) and their movements recorded for 2.5 min to determine baseline exploratory behaviour and locomotion (T1). During the second session (T2) the wire-mesh contained an unfamiliar CD1 aggressive mouse and the time the experimental C57BL/6 mouse spent in its proximity was measured. Trials were video-recorded and analysed by an experienced observer unaware of the group assignment to time spent in the interaction zone, that is 5 cm around the wire mesh cage. Exploration was defined as sniffing or touching the

cage with the nose and/or forepaws. Social interaction (SI) was calculated as the ratio between the time spent in the interaction zone during T2 and T1.

Behavioural sequence: experimental procedure, quantitative analysis T patterns and statistical analysis: The procedure is described in (Santangelo et al. 2017). Recording of mice behavioural components was conducted during the social interaction tests. The ethogram, which is a list containing the description of the behavioural elements of interest, was compiled and encompassed *Cage related* and *Other behaviours* as shown in Table7. Based on this ethogram, a trained researcher, blind to treatments, converted the continuous flow of the recorded behaviour in an event log file containing behavioural components and their time onset, by using a software coder (The Observer, Noldus Information Technology bv, The Netherlands). Measurements of frequency and duration of each component of the behavioural repertoire were based on the descriptive analysis shown in Table 8. T-pattern analysis was performed as in (Santangelo et al. 2017). This is a multivariate approach conceived to detect the time relationship among events. The software THEME, specifically developed for this purpose (Pattern-Vision, Ltd, Iceland) was used. By processing event log files obtained for each subject, THEME algorithm detects recurring sequences of events characterized by statistically significant constraints among the interval length separating them. THEME follows a bottom-up procedure based on the comparison among the distribution of each possible pair of events. As described in (Santangelo et al. 2017):'Assuming "A" and "B" as two behavioural components with a given distribution along the time window, the "A e B" pair is defined as "t-pattern" only if a statistically significant time interval between the two events exists. In this case such a t-pattern is indicated as "(A B)" and considered by the algorithm as a potential "A" or "B" term in higher order patterns, e.g. "((A B) C)". This procedure continues up to any level, to be completed when no more patterns are found.'

Here the results are presented as T-pattern strings, which are the textual description of a pattern and its hierarchical composition. Frequencies and durations are presented as mean number ± SE performed by each subject during the testing time.

Differences among groups were assessed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons.

To detect recurrent behavioural T-pattern analysis was employed (fore details please refer to (Santangelo et al. 2017).

Novel object recognition test: This protocol measures a form of memory based on short and unrepeated experiments without any reinforcement, such as food or electric shocks (Ennaceur and Delacour 1988). Mice were placed in a white polyvinylchloride box (70 × 60 cm and 30 cm high) with a grid floor that is easily cleaned and illuminated by a 75-W lamp suspended 50 cm above the box. The objects to be discriminated were gray polyvinyl chloride shapes: cubes of 8 cm side, pyramids and cylinders of 8 cm height. The object recognition task consisted of a training phase (T1) and a testing phase (T2). Twenty-four h prior to T1, mice were habituated for two 10 min-session to the experimental apparatus in the absence of any object. On the day of the experiment, the mouse was placed for 5 min into the test arena facing the same direction and in the same position in the presence of two identical plastic objects (T1). The behaviour of mice was videotaped, and the time spent actively exploring the objects was measured. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. T2 was performed 1 h after T1, during which, each mouse was again placed in the test arena for 5 min in the presence of one of the familiar objects and a novel object. The position of the objects (left/right) was randomized to prevent bias due to order or place preference. The behaviour of mice during T2 was videotaped, and the exploration time of the familiar (F) and the new object (N) were measured. To avoid place preference the position of the two objects during T2 was randomly changed.

Table 7. Ethogram qualitatively describing the behaviours (events) of mice during the social interaction test. These comprise events that in the proximity of the cage holding the CD1 mouse (Cage Related Events) and Other Events that occur in the arena distant from the encaged CD1 mouse.

ETHOGRAM		
CAGE RELATED EVENTS		
Cage Sniffing	**CS**	the mouse sniffs Cage borders and/or ground
Cage Leaning	**CL**	the mouse maintains an erect posture by leaning against Cage walls
Cage Climbing	**CC**	the mouse mounts on Cage walls and roof. At least three paws grab the Cage grid
Cage Retraction	**CR**	the mouse suddenly retracts its head-shoulder segment or its body far from the Cage.
NON CAGE RELATED (OTHER) EVENTS		
Walking	**Wa**	the mouse walks in the arena. Sniffing activities may be produced if locomotion continues
Place Sniffing	**PS**	the mouse sniffs the surrounding arena environment without walking activity. Head and vibrissae movements are produced. If the mouse sniffs the central Cage borders and/or ground the correct annotation is *Cage Sniffing*
Stretched Sniffing	**SS**	the mouse stretches its head and shoulders forward and then returns to the original position. Anterior limbs stand still
Wall Leaning	**WL**	the mouse maintains an erect posture by leaning against arena walls
Rearing	**Re**	the mouse maintains an erect posture without leaning against the walls
Jumping	**Ju**	the mouse leaps from the surface of the arena
Fore Body Grooming	**FBG**	The mouse licks or rubs its face and/or its anterior limbs
Hind Body Grooming	**HBG**	the mouse licks or rubs its body fur and/or its posterior limbs
Immobility	**Im**	the mouse maintains a fixed posture

Results

Mice of both $HDC^{+/+}$ and $HDC^{-/-}$ genotypes were subjected to the protocol shown in Figure 25. Non-stressed mice of either genotype were left undisturbed in their home cage until sacrifice. For clarity, results of $HDC^{+/+}$ and $HDC^{-/-}$ are shown separately.

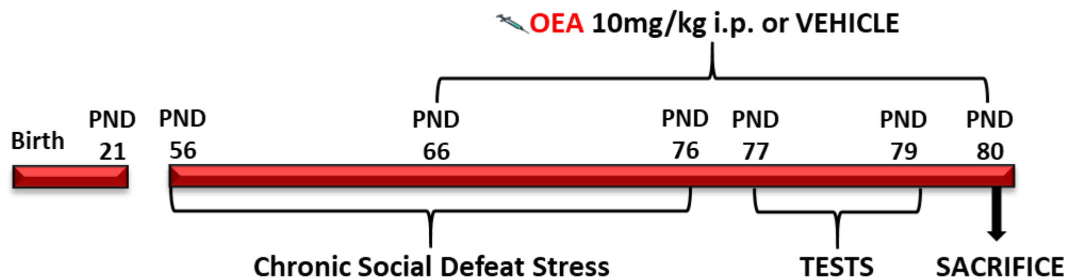


Figure 25. Timeline for the chronic social defeat stress experiment and OEA or vehicle injections. Mice were randomly assigned to three experimental groups per genotype

Stress and OEA did not affect body weight change

$HDC^{+/+}$ mice gained comparable weight as two-way ANOVA showed a significant effect of time, independent of treatment or stress ($F_{time\ (2.54)} = 77.30$; $P < 0.0001$; $F_{treatment\ (2.54)} = 0.3752$; $P = 0.6889$; $F_{interaction\ (4.54)} = 0.1983$; $P = 0.9382$; Figure 26A), and consumed similar quantities of food regardless of stress and treatment (Figure 26C; $F_{time\ (2.54)} = 1867$, $P < 0,0001$; $F_{treatment\ (2.54)} = 1.032$, $P = 0.3631$; $F_{interaction\ (4.54)} = 1.682$, $P = 0.1675$). $HDC^{-/-}$ mice as well gained comparable weight during the 21 days of stress regardless of treatment ($F_{time\ (2.48)} = 137.9$, $P < 0.0001$; $F_{treatment\ (2.48)} = 0.8536$, $P = 0.4322$; $F_{interaction\ (4.48)} = 0.7076$, $P = 0.5907$). A two-way ANOVA showed an overall significant difference in food consumption among $HDC^{-/-}$ mice ($F_{Interaction\ (4.48)} = 20.54$, $P < 0.0001$; $F_{time\ (2.48)} = 1800$, $P < 0.0001$; $F_{treatments\ (2.48)} = 35.69$ $P < 0.0001$). Bonferroni's post hoc analysis revealed that stressed mice ate significantly more than non-stressed mice regardless of the treatment (Figure 26D).

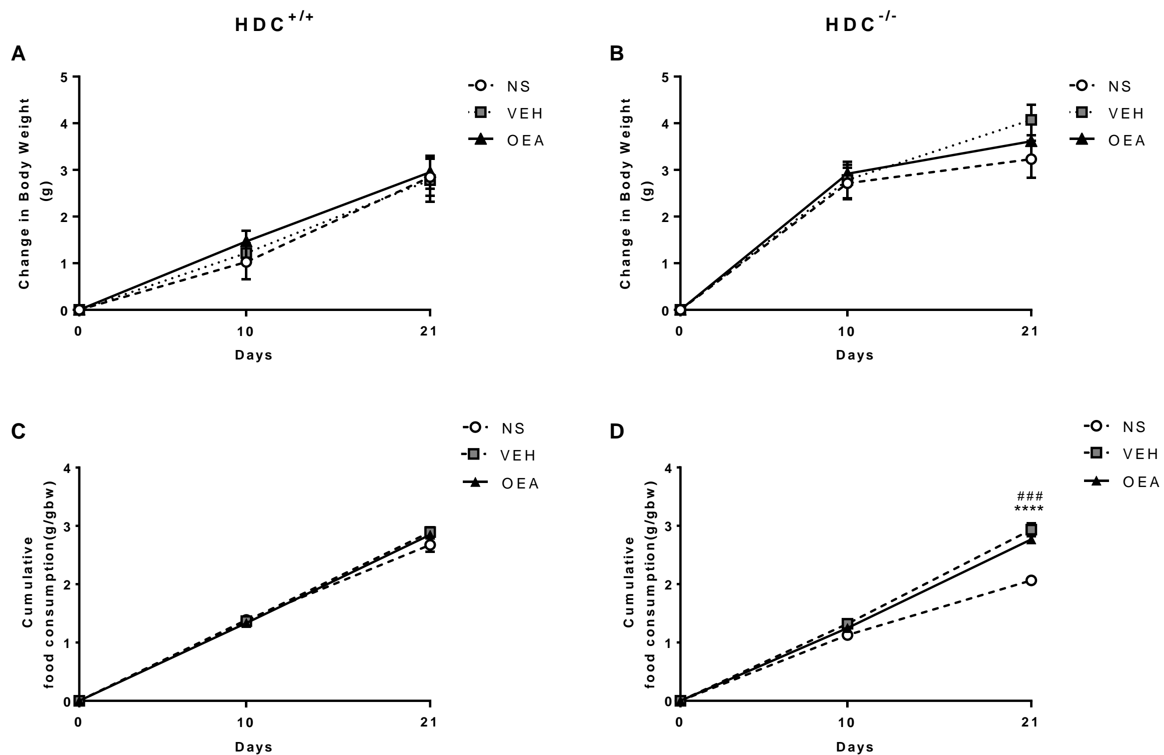


Figure 26. Effects of stress and OEA (10 mg/kg) on weight gain at T0 before initiating stress protocol, at T10 before starting OEA treatment, at T21 on completion of the stress procedure. N=6-8 experimental group. Data are shown as means ± S.E.M. **** P < 0.0001 VEH vs NS; ### P < 0.001 OEA vs NS, by two-way ANOVA and the Bonferroni's post hoc test.

OEA prevented social aversion induced by stress only in HDC$^{+/+}$ mice

The genetic lack of histamine did not affect sociability, as non-stressed mice of either genotype interacted similarly with the caged CD1 mouse, as shown in Figure 27 A and B. One-way ANOVA revealed that CSDS affected the behaviour of both HDC$^{+/+}$ ($F_{(2, 21)} = 15.57$ $P < 0.0001$) and HDC$^{-/-}$ mice ($F_{(2, 17)} = 61.26$, $P < 0.0001$), as the total time that mice spent in the proximity of the caged CD1 mouse was significantly shorter than that of non-stressed mice. OEA though, partially prevented the social aversion of HDC$^{+/+}$ but did not change the behaviour of stressed HDC$^{-/-}$ mice.

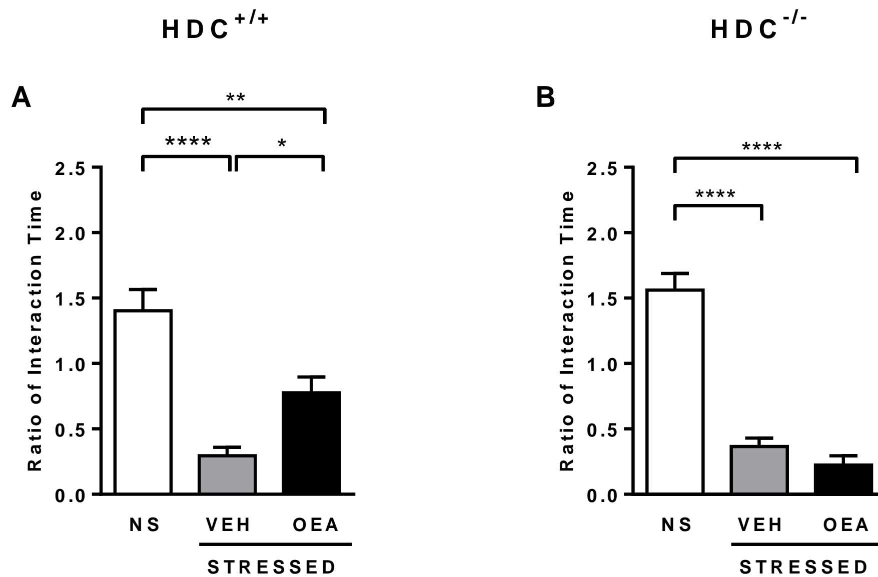


Figure 27. Effect of OEA administration on social-avoidance behaviour induced by stress. Repeated social defeat stress induced social avoidance of both HDC$^{+/+}$ and HDC$^{-/-}$ mice expressed as the ratio of time a mouse spent in the interaction zone in the presence of a target CD-1 compared with the absence of a target CD-1. OEA partially prevented social avoidance of HDC$^{+/+}$ mice, but not that of HDC$^{-/-}$ mice. Data are presented as means ± S.E.M. n = 6-9/group ****P < 0.0001, **P < 0.01 vs NS; $^{\#}$P < 0.05 vs Veh by one-way ANOVA and the Neumann Keuls' post hoc test

OEA restored the behavioural patterns impaired by stress in HDC$^{+/+}$ but not in HDC$^{-/-}$ mice

Stress and OEA did not affect the locomotion of mice of either genotype as the time spent in the central or peripheral zone of an empty arena was not affected by stress nor treatment (Figure 28). Despite the gross similarity of behaviours between non-stressed HDC$^{+/+}$ and HDC$^{-/-}$ mice, we know that brain histamine contributes to the qualitative features of displayed motor behaviours not only in animals (Santangelo et al. 2017), but also in humans (Baldan et al. 2014).

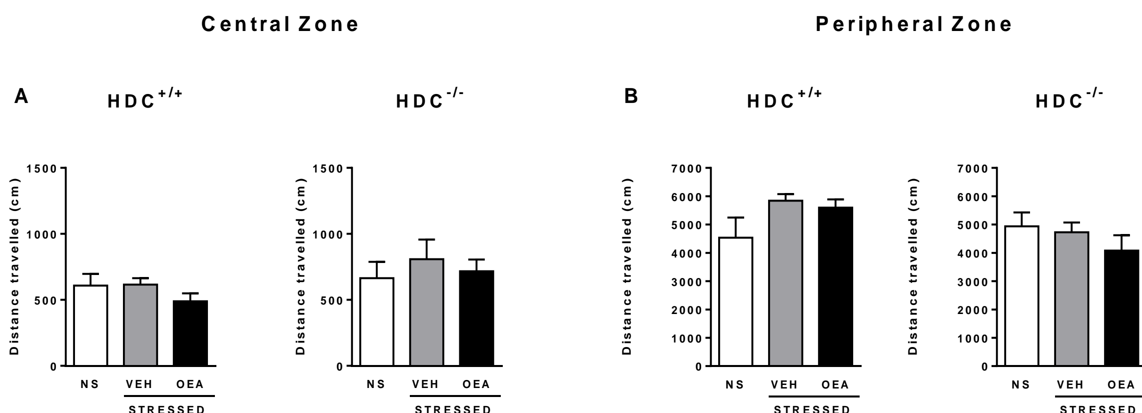


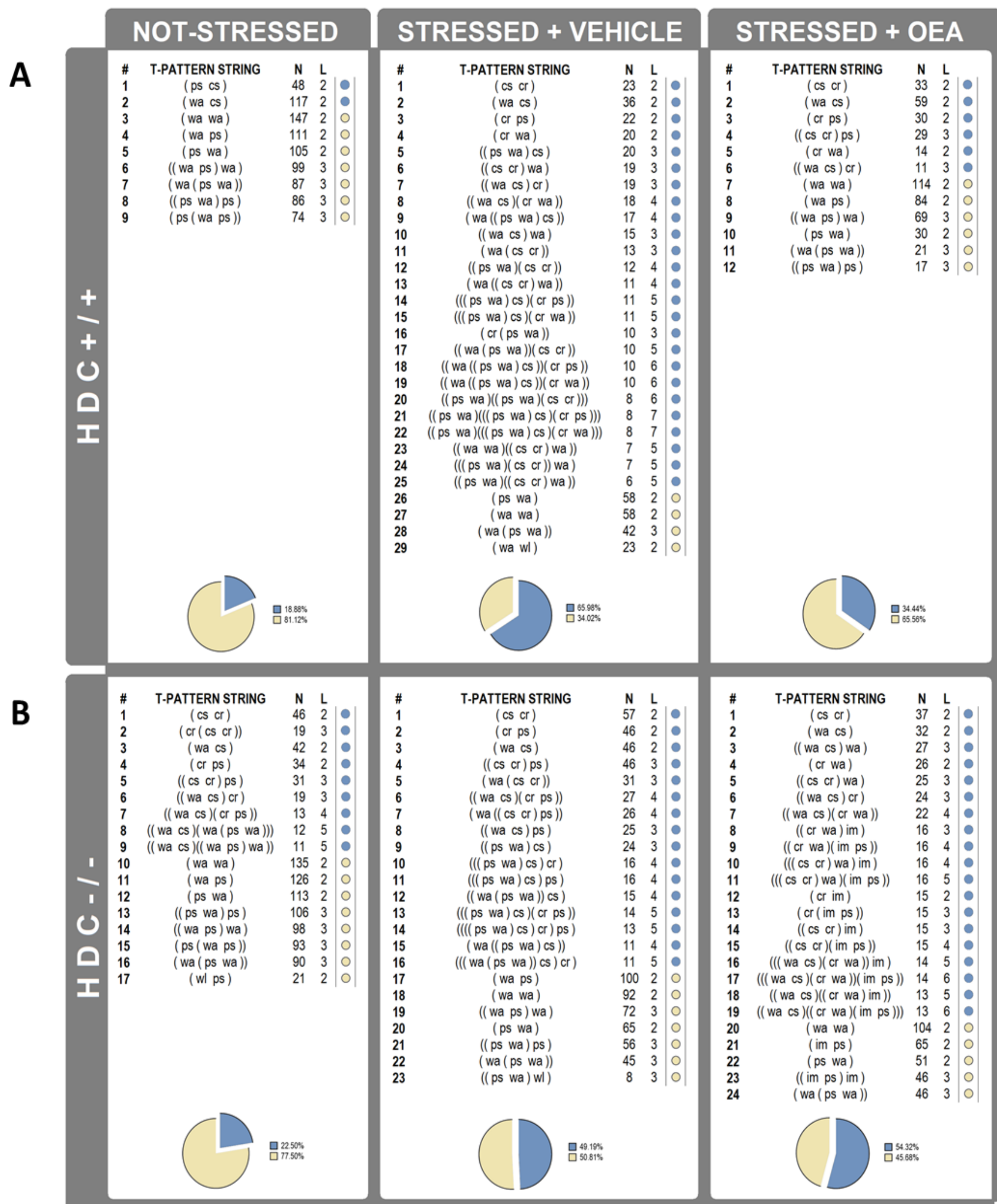
Figure 28. Chronic social defeat stress did not alter locomotor activity in an open field arena. Distance travelled in the (A) centre of the arena and in the (B) peripheral zone.

Therefore, we analysed the complex behavioural sequence of experimental mice during the social interaction test. The quantitative analysis was based on an ethogram that encompassed *Cage related* (i.e. in the proximity of the cage holding the CD1 mouse) and *Non cage related* (*Other) events* (i.e. displayed in the arena; Table 7). Mean (± SE) numbers and duration of each specific component of the ethogram are shown in Table 8. The statistical analysis was possible only for the sufficiently represented behaviours.

Cage sniffing was by far the most frequent and long-lasting behaviour among *Cage Related* components and was performed by both HDC$^{+/+}$ and HDC$^{-/-}$ mice. ANOVA showed a statistically significant effect of treatments on mean occurrence and mean duration only among HDC$^{+/+}$ groups. Stressed HDC$^{+/+}$ mice treated with vehicle sniffed the cage less frequently and for a much shorter time than non-stressed mice, and OEA significantly improved both parameters. On the other hand, chronic stress, despite decreasing significantly the time of social interaction (Figure 27B), did not impact on Cage Sniffing occurrence and duration in HDC$^{-/-}$ mice at a statistical level. Nor OEA appreciably affected these parameters (Table 8). Walking and place sniffing were the most frequent *Other events* of both genotypes. The mean Walking occurrence of HDC$^{+/+}$ mice changed with stress and stress + OEA although the results did not reach statistical significance; however, the mean duration of Place sniffing was significantly longer in stressed HDC$^{+/+}$ mice treated with either vehicle or OEA. The quantitative analysis of HDC$^{-/-}$ mice behaviour was overall different from HDC$^{+/+}$ mice. No significant differences were found in the number or duration of *Cage Related* and *Other events* among HDC$^{-/-}$ experimental groups (Table 8). The T-pattern analysis is conceived to detect events in time-ordered sequences characterized by statistically significant constraints among them (Casarrubea et al., 2015); (Santangelo et al., 2017). Mice behavioural structure was characterized by a complex temporal organization in the arena during the social interaction. Nine T- attern strings were detected in non-stressed HDC$^{+/+}$ encompassing 2 or 3 events in their structure, with only 2 of them containing *Cage Related* events, namely "(ps cs)" and "(wa cs)" (Figure 29A, blue dots, see Table 7 for abbreviations). The stressed group showed a strikingly more complex behavioral structure, as HDC$^{+/+}$ mice performed a total of 29 T-pattern strings, 25 of which containing C*age Related Events*, mostly Cage Sniffing followed by Cage Retraction (cr). The *Other Events* comprised mainly Walking and Place Sniffing (yellow dots).

Stressed HDC$^{+/+}$ mice treated with OEA performed 12 T-pattern strings very similar in structure to non-stressed mice, although *Cage Related Events* within each pattern were more numerous (6 out of 12, exclusively Cage Sniffing and Cage Retraction).

Non-stressed $HDC^{-/-}$ mice showed a strikingly more complex behavioural structure compared to non-stressed $HDC^{+/+}$ mice (Figure 29B), similar to that observed in CD1 mice pharmacologically deprived of histamine (Santangelo et al., 2017). Non-stressed $HDC^{-/-}$ mice displayed 17 T-pattern strings encompassing up to 5 events out of which 9 contained a *Cage Related Event* (Figure 29B; blue dots). Stress increased considerably the number of complex patterns of $HDC\text{-}^{/-}$ mice as well, in particular T-patterns containing *Cage Related Events* (16 out of 23). Oleoylethanolamide did not significantly change the total number of T-pattern strings, but further increased the complexity up to 6 events per string containing Immobility, Place Sniffing and Walking, but did not change considerably the number of patterns containing *Cage Related Events*. The pies in Figure 29 represent the percentage of *Cage Related* (blue) and *Other* T-patterns (yellow) displayed by each experimental group.



A — HDC +/+

NOT-STRESSED

#	T-PATTERN STRING	N	L	
1	(ps cs)	48	2	●
2	(wa cs)	117	2	●
3	(wa wa)	147	2	○
4	(wa ps)	111	2	○
5	(ps wa)	105	2	○
6	((wa ps) wa)	99	3	○
7	(wa (ps wa))	87	3	○
8	((ps wa) ps)	86	3	○
9	(ps (wa ps))	74	3	○

STRESSED + VEHICLE

#	T-PATTERN STRING	N	L	
1	(cs cr)	23	2	●
2	(wa cs)	36	2	●
3	(cr ps)	22	2	●
4	(cr wa)	20	2	●
5	((ps wa) cs)	20	3	●
6	((cs cr) wa)	19	3	●
7	((wa cs) cr)	19	3	●
8	((wa cs)(cr wa))	18	4	●
9	(wa ((ps wa) cs))	17	4	●
10	((wa cs) wa)	15	3	●
11	(wa (cs cr))	13	3	●
12	((ps wa)(cs cr))	12	4	●
13	(wa ((cs cr) wa))	11	4	●
14	(((ps wa) cs)(cr ps))	11	5	●
15	(((ps wa) cs)(cr wa))	11	5	●
16	(cr (ps wa))	10	3	●
17	((wa (ps wa))(cs cr))	10	5	●
18	((wa ((ps wa) cs))(cr ps))	10	6	●
19	((wa ((ps wa) cs))(cr wa))	10	6	●
20	((ps wa)((ps wa)(cs cr)))	8	6	●
21	((ps wa)(((ps wa) cs)(cr ps)))	8	7	●
22	((ps wa)(((ps wa) cs)(cr wa)))	8	7	●
23	((wa wa)((cs cr) wa))	7	5	●
24	(((ps wa)(cs cr)) wa)	7	5	●
25	((ps wa)((cs cr) wa))	6	5	●
26	(ps wa)	58	2	○
27	(wa wa)	58	2	○
28	(wa (ps wa))	42	3	○
29	(wa wl)	23	2	○

STRESSED + OEA

#	T-PATTERN STRING	N	L	
1	(cs cr)	33	2	●
2	(wa cs)	59	2	●
3	(cr ps)	30	2	●
4	((cs cr) ps)	29	3	●
5	(cr wa)	14	2	●
6	((wa cs) cr)	11	3	●
7	(wa wa)	114	2	○
8	(wa ps)	84	2	○
9	((wa ps) wa)	69	3	○
10	(ps wa)	30	2	○
11	(wa (ps wa))	21	3	○
12	((ps wa) ps)	17	3	○

B — HDC -/-

NOT-STRESSED

#	T-PATTERN STRING	N	L	
1	(cs cr)	46	2	●
2	(cr (cs cr))	19	3	●
3	(wa cs)	42	2	●
4	(cr ps)	34	2	●
5	((cs cr) ps)	31	3	●
6	((wa cs) cr)	19	3	●
7	((wa cs)(cr ps))	13	4	●
8	((wa cs)(wa (ps wa)))	12	5	●
9	((wa cs)((wa ps) wa))	11	5	●
10	(wa wa)	135	2	○
11	(wa ps)	126	2	○
12	(ps wa)	113	2	○
13	((ps wa) ps)	106	3	○
14	((wa ps) wa)	98	3	○
15	(ps (wa ps))	93	3	○
16	(wa (ps wa))	90	3	○
17	(wl ps)	21	2	○

STRESSED + VEHICLE

#	T-PATTERN STRING	N	L	
1	(cs cr)	57	2	●
2	(cr ps)	46	2	●
3	(wa cs)	46	2	●
4	((cs cr) ps)	46	3	●
5	(wa (cs cr))	31	3	●
6	((wa cs)(cr ps))	27	4	●
7	(wa ((cs cr) ps))	26	4	●
8	((wa cs) ps)	25	3	●
9	((ps wa) cs)	24	3	●
10	(((ps wa) cs) cr)	16	4	●
11	(((ps wa) cs) ps)	16	4	●
12	((wa (ps wa)) cs)	15	4	●
13	(((ps wa) cs)(cr ps))	14	5	●
14	((((ps wa) cs) cr) ps)	13	5	●
15	(wa ((ps wa) cs))	11	4	●
16	(((wa (ps wa)) cs) cr)	11	5	●
17	(wa ps)	100	2	○
18	(wa wa)	92	2	○
19	((wa ps) wa)	72	3	○
20	(ps wa)	65	2	○
21	((ps wa) ps)	56	3	○
22	(wa (ps wa))	45	3	○
23	((ps wa) wl)	8	3	○

STRESSED + OEA

#	T-PATTERN STRING	N	L	
1	(cs cr)	37	2	●
2	(wa cs)	32	2	●
3	((wa cs) wa)	27	3	●
4	(cr wa)	26	2	●
5	((cs cr) wa)	25	3	●
6	((wa cs) cr)	24	3	●
7	((wa cs)(cr wa))	22	4	●
8	((cr wa) im)	16	3	●
9	((cr wa)(im ps))	16	4	●
10	(((cs cr) wa) im)	16	4	●
11	(((cs cr) wa)(im ps))	16	5	●
12	(cr im)	15	2	●
13	(cr (im ps))	15	3	●
14	((cs cr) im)	15	3	●
15	((cs cr)(im ps))	15	4	●
16	(((wa cs)(cr wa)) im)	14	5	●
17	(((wa cs)(cr wa))(im ps))	14	6	●
18	((wa cs)((cr wa) im))	13	5	●
19	((wa cs)((cr wa)(im ps)))	13	6	●
20	(wa wa)	104	2	○
21	(im ps)	65	2	○
22	(ps wa)	51	2	○
23	((im ps) im)	46	3	○
24	(wa (ps wa))	46	3	○

Figure 29. Effect of stress and OEA on T-patterns of HDC$^{+/+}$ mice. A) T-pattern string = textual representation of each pattern of different composition; brackets indicate the hierarchical structure. N = occurrences of patterns of different composition. L = number of patterns within each string. Pies represent percent distribution of T-patterns containing *Cage Related* (blue) and *Other Events* (yellow). B) Effect of stress and OEA on T-patterns of HDC$^{-/-}$ mice. T-pattern string = textual representation of each pattern of different composition; brackets indicate the hierarchical structure. N = occurrences of patterns of different composition. L = number of patterns within each string. B) Pies represent percent distribution of T-patterns containing *Cage Related* (blue) and *Other Events* (yellow).

Table 8. The table lists the occurrence and duration of each event in the total population of mice divided by genotype and treatment.

HDC +/+

Behav.	OCCURRENCE								DURATION							
	NS		VEH		OEA		F	*p*	NS		VEH		OEA		F	*p*
	Mean (n)	SE	Mean	SE	Mean	SE			Mean (s)	SE	Mean	SE	Mean	SE		
cs	31.00	1.95	9.20	1.93	18.00	5.32	**10.068**	**0.003**	60.41	4.23	15.86	5.19	30.40	9.61	**11.293**	**0.002**
cl	5.60	1.63	0.40	0.24	1.00	0.63	-	-	11.84	4.44	2.76	0.80	4.80	0.64	-	-
cc	0.80	0.37	0.00	0.00	0.00	0.00	-	-	6.28	1.83	0.00	0.00	0.00	0.00	-	-
cr	3.40	2.93	4.80	0.73	6.80	1.16	0.839	0.456	2.34	1.46	1.41	0.21	2.76	0.62	1.648	0.245
wa	42.40	3.04	25.60	2.98	28.20	7.74	3.143	0.080	38.52	4.06	26.50	2.46	26.63	6.40	2.252	0.148
ps	27.40	1.96	26.80	1.66	30.40	3.16	0.674	0.528	20.87	2.51	69.34	10.41	57.20	12.85	**6.816**	**0.011**
ss	3.20	1.11	1.60	0.81	1.00	0.55	-	-	2.57	0.92	3.09	0.29	1.11	0.45	-	-
wl	4.00	1.48	5.20	1.74	3.60	1.17	0.315	0.736	9.96	3.08	9.24	4.33	5.76	1.15	0.408	0.675
re	2.20	1.96	1.80	1.20	0.40	0.24	-	-	4.74	4.38	3.56	1.52	0.58	0.42	-	-
ju	0.00	0.00	0.40	0.40	0.00	0.00	-	-	0.00	0.00	2.16	0.00	0.00	0.00	-	-
fbg	1.00	0.77	1.80	0.58	4.40	1.29	-	-	4.24	1.80	5.66	1.43	6.84	2.53	-	-
hbg	0.00	0.00	0.40	0.24	1.00	1.00	-	-	0.00	0.00	1.16	0.40	6.28	0.00	-	-
im	0.00	0.00	6.60	2.69	7.00	2.72	-	-	0.00	0.00	27.80	2.31	23.52	7.93	-	-

HDC -/-

Behav.	OCCURRENCE								DURATION							
	NS		VEH		OEA		F	*p*	NS		VEH		OEA		F	*p*
	Mean (n)	SE	Mean	SE	Mean	SE			Mean (s)	SE	Mean	SE	Mean	SE		
cs	16.00	1.57	14.67	4.14	8.67	2.79	1.673	0.221	25.25	1.71	20.09	4.69	12.53	4.73	2.601	0.107
cl	0.67	0.49	0.50	0.34	0.17	0.17	-	-	3.30	2.58	3.48	1.96	2.52	0.00	-	-
cc	0.00	0.00	0.00	0.00	0.00	0.00	-	-	0.00	0.00	0.00	0.00	0.00	0.00	-	-
cr	8.17	0.60	9.50	2.93	6.33	1.94	0.596	0.564	3.28	0.64	3.03	1.05	1.65	0.70	1.167	0.338
wa	29.50	4.42	25.50	4.19	22.00	5.16	0.663	0.530	34.28	5.44	26.14	3.90	21.15	5.53	1.749	0.208
ps	31.83	2.77	28.00	2.53	26.50	3.59	0.840	0.451	65.83	6.69	69.62	7.49	71.11	8.14	0.133	0.877
ss	3.17	0.79	1.67	0.76	0.83	0.31	-	-	3.04	0.67	1.83	0.42	1.51	0.33	-	-
wl	5.67	1.63	3.83	1.08	2.33	1.17	1.613	0.232	8.46	2.74	6.75	2.00	4.37	2.24	0.720	0.504
re	0.00	0.00	0.33	0.33	0.00	0.00	-	-	0.00	0.00	0.92	0.00	0.00	0.00	-	-
ju	0.00	0.00	0.00	0.00	0.00	0.00	-	-	0.00	0.00	0.00	0.00	0.00	0.00	-	-
fbg	2.83	0.87	2.67	0.99	0.50	0.34	-	-	7.26	1.17	6.07	1.59	5.82	0.18	-	-
hbg	0.33	0.33	0.33	0.33	0.00	0.00	-	-	3.08	0.00	1.16	0.00	0.00	0.00	-	-
im	2.00	1.29	6.33	2.01	11.00	1.97	-	-	5.32	1.90	19.85	7.27	36.52	8.15	-	-

Figure 30 shows the mean number of T-patterns performed by each mouse with statistical significance. One-way ANOVA of $HDC^{+/+}$ mice showed significant difference among groups (F2,14 =13.980; P < 0.001). Bonferroni MCT showed that on average, stressed $HDC^{+/+}$ mice performed significantly more behavioural patterns containing *Cage Related Events*, whereas stressed $HDC^{+/+}$ treated with OEA did not differ significantly from non-stressed mice (Figure 30A). The opposite was true for T-pattern strings containing *Other Events* ($F_{(2,14)}$= 21.203; P < 0.001) although OEA only partially prevented the effect of stress on this parameter (Figure 30B). No statistically significant differences of T-pattern strings containing *Cage Related Events* were found among $HDC^{-/-}$ mice of all experimental groups ($F_{(2,17)}$= 1.365; P > 0.05; Figure 30C). One-way ANOVA of T-pattern mean number not containing *Cage Related Events* of stressed $HDC^{-/-}$ mice found significant differences among groups ($F_{(2,17)}$ = 6.136; P < 0.05; Figure 30D). Bonferroni's MCT showed that mice treated with OEA performed significantly less T-patterns containing *Other Events*.

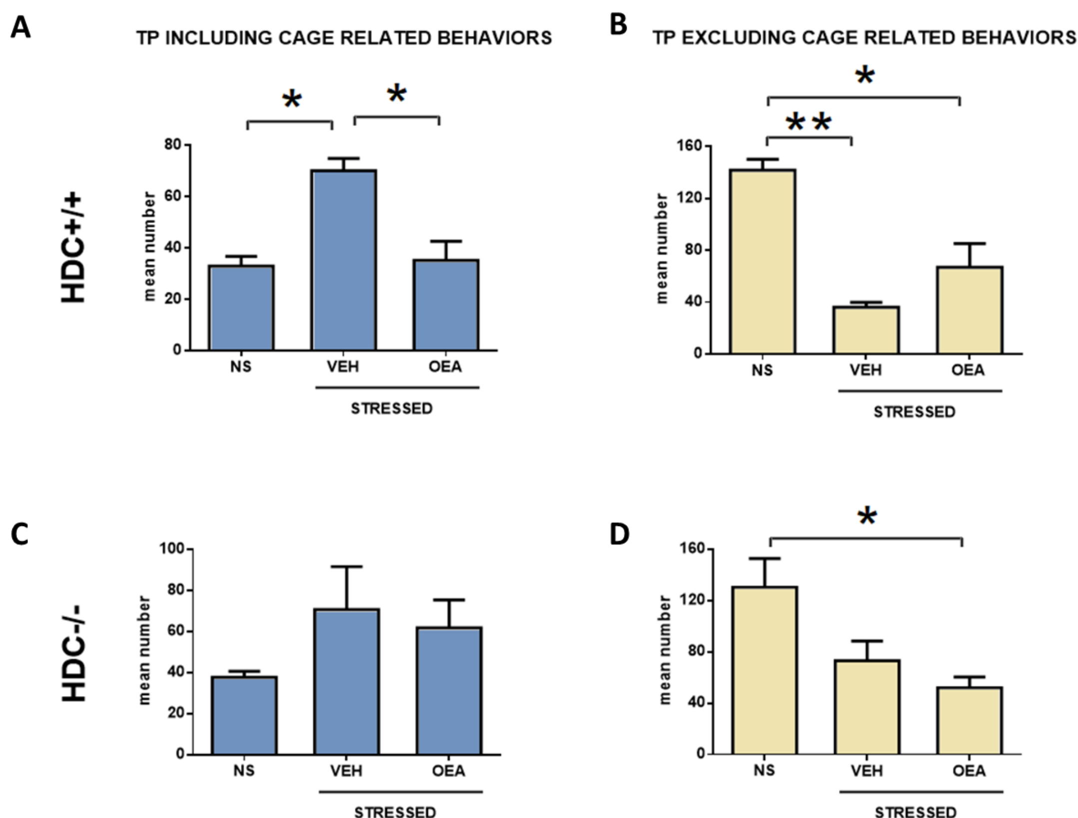


Figure 30. T-pattern chart. Results were obtained from T-pattern analysis and represent the mean number ± SE of T-pattern strings of different composition for each experimental group during the social interaction test. (A) and B) T-pattern strings including and excluding cage related events, respectively, of $HDC^{+/+}$ mice.

OEA prevented the memory impairment induced by chronic stress

Figure 31 shows the performance of mice in the novel object recognition test. Two-way ANOVA showed a statistical difference among HDC$^{+/+}$ experimental groups (treatments $F_{(2, 34)} = 9.746e\text{-}015$ $P > 0.9999$; objects $F_{(1, 34)} = 19.56$ $P < 0.0001$; interaction $F_{(2, 34)} = 2.604$ $P = 0.0887$) and HDC$^{-/-}$ mice (treatments $F_{(2, 32)} = 3.576e\text{-}014$ $P > 0.9999$; objects $F_{(1, 32)} = 1.231$ $P = 0.2754$; interaction $F_{(2, 32)} = 5.115$ $P = 0{,}0118$). When tested 1 hr after training, both HDC$^{+/+}$ and HDC$^{-/-}$ mice spent significantly more time exploring the new object demonstrating a good memory of the familiar one. Three weeks of CSDS had a negative effect on mice ability to discriminate between the familiar and new object, which indicates that stressed mice of both genotypes had a cognitive impairment. OEA treatment rescued the behavioural impairment of HDC$^{+/+}$ mice only, as OEA-treated HDC$^{-/-}$ mice did not show any memory improvement.

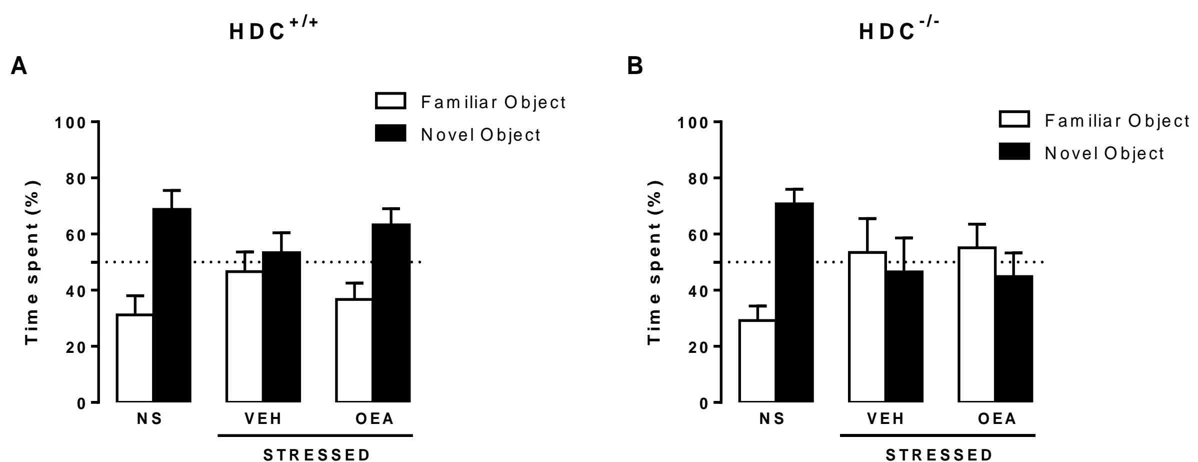


Figure 31. Effect of OEA administration on stress-induced cognitive impairment in the novel object recognition test. Social defeat stress affected the performance of both HDC$^{+/+}$ and HDC$^{-/-}$ mice when tested 1 h after training which was prevented by OEA administration to HDC$^{+/+}$, but not to HDC$^{-/-}$ mice. N = 6-8/group **P < 0.01 vs familiar object by two-way ANOVA and the Bonferroni's post hoc test.

Conclusion: Part II

Chronic uncontrollable stress is a major risk factor for the development of metabolic and psychiatric disorders. A useful preclinical model to understand the molecular mechanisms underlying affective-like disorders is the social defeat stress which results in the development of depressive-like behavioural impairments characterized by enduring deficits in metabolic processes (van der Kooij et al. 2018), social interactions (Golden et al. 2011) and memory (Monleón, Duque and Vinader-Caerols 2016).

In this second part of my PhD thesis first of all I investigated the effects of the ω-3 PUFA and Vitamin A supplanted diet on stress-induced cognitive and neurochemical changes induced by chronic social defeat stress focusing my attention on the role of the central histaminergic system.

We found that the ω-3 PUFA and Vitamin A enriched diet reduces social avoidance, improves recognition memory in the NOR test and spatial memory in the NOL test of mice subjected to 10 days of chronic social defeat stress (CSDS). Moreover, we observed that ω-3 PUFA and Vitamin A enriched diet increased synaptophysin expression in the hippocampus of normal mice which is in accord to the literature (Hajjar et al. 2013).

The novelty of this study consists in using a chronic stress protocol with ethological significance to investigate the effectiveness of the enriched diet, and primarily, the inefficacy of the diet to prevent the deleterious, stress-induced cognitive effects in mice unable to synthesize histamine. Vitamin A and ω-3 PUFAs may modulate cerebral plasticity and memory by regulating gene expression through nuclear receptors that function as ligand-controlled transcription factors (Lane and Bailey 2005, Su 2010). We therefore explored a possible mechanism that makes the enriched diet work in $HDC^{+/+}$ but not in $HDC^{-/-}$ mice. We investigated the impact of stressful aggressive encounters on fatty acid metabolic enzymes mRNA expression within the hippocampus (HIP) and prefrontal cortex (PFC) in $HDC^{+/+}$ and $HDC^{-/-}$ mice fed a control diet or a diet supplemented with ω3-PUFA and vitamin A. Our results show that EPA/DHA and vitamin A supplemented diet produces an increase in the 12-LOX expression in the prefrontal cortex of $HDC^{+/+}$ mice fed a supplemented diet compared to $HDC^{-/-}$ animal fed with the same diet. This indicates that the histaminergic system is necessary for the enriched diet to exert its effects in the hippocampus and cortex by increasing the gene expression of an enzyme (12-LOX) which leads to the production of anti-inflammatory molecules. In the PFC we observed an increase in 12-LOX expression in $HDC^{+/+}$ and $HDC^{-/-}$ stressed animals fed with supplemented diet compared to non-stressed control group.

The increase in mRNA expression of the 12-LOX enzyme produces an incremented synthesis of 12-HETE which promote the activation of PPARγ that is neuroprotective through its anti-inflammatory properties (Shalini et al. 2018).

Recently, it was reported that intracerebroventricular treatment with resolvin D1, D2, E1, E2 and E3, which are derived from ω3-PUFA through the 5-LOX and 12-LOX enzymes, and infusion of these lipids to the PFC and hippocampus ameliorates depressive-like behaviours induced by bacterial endotoxin (Deyama et al. 2017, Deyama et al. 2018b, Deyama et al. 2018a). The beneficial effects of resolvin D1 and D2 were also demonstrated in a mouse model of chronic mild stress (Ishikawa et al. 2017).

Due to the sanitary emergency, the results are as off today incomplete. In collaboration with Dr. Layè of University of Bordeaux we are performing a lipidomic analysis in this two brain regions involved in mnemonic processes such as hippocampus and prefrontal cortex to evaluate any differences in lipid and their derivates oxylipins concentration that may explain, at least in part, the effect of the enriched diet in normal animals but not in histamine depleted animals in order to better understand the role of the central histaminergic system in this effect.

Previous work in our laboratory showed how the central histaminergic system is essential for the central actions of a lipid derivate, olelylethalamide (OEA). In fact, OEA requires the integrity of the central histaminergic system to exert its hypofagic (Provensi et al. 2014) , procognitive (Provensi et al. 2017) and antidepressant-like effects (Costa et al. 2018).

Despite being a valuable tool in drug discovery for high-throughput screening of prospective anti-depressants, the tail suspension test is inadequate to investigate the neurobiological substrates of chronic stress and the pathogenesis of mood disorders. There are several models of chronic stress and anxiety, including chronic social defeat (Krishnan et al. 2007). For this reason, in the second part of this section we investigated the effect of a fatty acid’s derived OEA treatment in chronic social defeat stress-induced behavioural deficits. The main finding of this project is the observation that repeated daily treatment with OEA prevented social interaction deficit short-term memory impairment and changes in the patterns of mice behaviours during social recognition, induced by chronic social stress. Furthermore, OEA’s effects required the integrity of the histaminergic system. We performed an in-depth investigation of dynamic behaviours by using ethograms and t-pattern analysis, which is being applied also to the study of patients affected by movement and behavioural disorders (Aiello et al. 2020). The T-pattern strings recorded during the social interaction, revealed a more complex picture than predicted by the quantitative evaluations of social interaction *per se*. We observed

several differences between non-stressed HDC$^{+/+}$ and HDC$^{-/-}$ mice and differences in the impact of stress and OEA on the two genotypes. First of all, non-stressed HDC$^{-/-}$ mice showed a marked increase in pattern complexity (T-pattern strings) with respect to HDC$^{+/+}$ mice, that involved both *Cage Related* and *Other Events*. The absence of histamine in the brain profoundly affected how single components of the T-pattern strings interacted in time, contributing to the configuration of repetitive patterns. A similar increased complexity and number of T-pattern strings was observed also in CD1 mice pharmacologically deprived of histamine with i.c.v. injections of α-fluoromethylhistidine, a suicide inhibitor of histidine decarboxylase (Santangelo et al. 2017).

When exposed to chronic social stress, HDC$^{+/+}$ mice sniffed the cage containing the aggressive mouse far less often and for shorter bouts (*cs* in table 8). However, stressed HDC$^{+/+}$ mice displayed increased complexity and number of T-pattern strings encompassing *Cage Related Events*. We interpret this as indicative of a conflict between an engrained approaching behaviour and fear caused by the presence of the aggressive mouse. In other words, HDC$^{+/+}$ mice displayed more varied T-pattern strings in their composition and containing a higher number of *Cage Related Events* suggesting a remarkable reorganization of the anxiety-related behaviour. A similar reorganization of anxiety related behaviours was observed after chronic administration of low doses of nicotine in rats (Casarrubea et al. 2020). Oleoylethanolamide partially restored the behavioural sequence, similar to that displayed by non-stressed mice, in terms of T-pattern string complexity, mean number of T-patterns (both *Cage Related and Other Events)*. In our paradigm, OEA seems to alleviate an anxiety-like behaviour induced by repeated social stress in mice. Interestingly, OEA decreased stressed induced binge-eating in female rats (Romano et al. 2020), supporting the pharmacological potential of OEA for the treatment of stress-related disorders.

The social interaction ratio of stressed HDC$^{-/-}$ mice was not dissimilar from HDC$^{+/+}$ mice, although T-pattern strings containing *Cage Related Events* were less numerous (16 vs 25), as well as the less complex (up to 7 components in HDC$^{+/+}$ mice and up to 5 in HDC$^{-/-}$). Furthermore, the mean frequency and duration of each component was not statistically different among stressed and non-stressed HDC$^{-/-}$ mice regardless of the pharmacological treatment. Apparently HDC$^{-/-}$ mice do remember the encounters with the aggressive CD1 mouse, as shown by the social interaction index, but the repertoire and duration of their behaviours, along with the T-pattern strings are completely different from HDC$^{+/+}$ mice.

Stressed HDC$^{-/-}$ mice treated with OEA showed no significant differences from vehicle-treated, stressed mice. OEA did not modulate the behavioural repertoire of histamine deficient mice.

$HDC^{-/-}$ mice apparently have a dysregulated striatal and prefrontal cortex function (Rapanelli et al. 2017a, Rapanelli et al. 2017b, Santangelo et al. 2017) that may contribute to the aberrant behaviours and memory impairment of these mice and the lack of response to OEA. Indeed, CSDS also compromised the short-term memory of both $HDC^{+/+}$ and $HDC^{-/-}$ mice, and the procognitive effects of OEA (Campolongo et al. 2009) were lost in histamine-deficient mice.
Considering these results, we suggest that peripheral signals generated by both the diet and OEA converge onto the central histaminergic system that provides the necessary central signalling to prevent stress-induced cognitive deficits and social aversion.

Part III: Brain histamine is necessary for long-term but not short-term social memory

For gregarious animals that live in societies or groups (like rodents), social memory is crucial to remember and recognize different conspecific individuals (i.e. having social memory) in order to exhibit the appropriate social behaviour such as aggression, avoidance, cooperative behaviour, and even mating behaviour (t. 2018, Okuyama 2018). Many neurotransmitters and hormones have been suggested to play key roles in social discrimination. Current evidence indicates that also the central histaminergic system modulates social recognition learning, however not much is known about the specific phases regulated by neuronal histamine (Provensi et al. 2018a). In this last part of my thesis work we investigate the impact of histaminergic neurotransmission deficiency or potentiation in short and long-term social recognition memory.

Materials and methods

Animals and Drug: Normal and histamine-deficient adult (8-9 weeks old) and juvenile (21-30 days old) male mice were used. Adult mice were used to perform the experiment and juvenile mice were used as social stimulus. Mice were housed in humidity and temperature-controlled room (22–24 °C) in the Centro Stabulazione Animali da Laboratorio (CeSAL), Università di Firenze. Mice were allowed free access to food (4RF21; Mucedola s.r.l., Italy) and water, and kept on a 12-h light/dark cycle (lights start at 7:00 a.m.). All the experiments were conducted between 9:00 a.m. and 4:00 p.m. Genotypes were confirmed using the PCR protocol according to (Provensi et al. 2014). Housing, animal maintenance and all experiments were conducted in accordance with the Council Directive of the European Community (2010/63/EU) and the Italian Decreto Legislativo 26 (13/03/2014), NIH guidelines on animal care and approved and supervised by a veterinarian.

Reduction of brain histamine levels was achieved using three different approaches: (i) mice lacking the HDC gene ($HDC^{-/-}$); (ii) i.c.v.) injections of the HDC inhibitor alpha-fluoromethylhistidine (α-FMH) and (iii) administration of the brain permeant H_3 receptor agonist VUF16839 (5mg/kg, i.p.). We used $HDC^{+/+}$ and $HDC^{-/-}$ mice treated with an H_3 receptor antagonist, Ciproxifan (3mg/kg, i.p.) to increase histamine levels. Moreover, we use a last group treated with both VUF16839 (5mg/kg, i.p.) and Donepezil (3mg/kg, i.p.)

Treated mice and their respective controls were evaluated in the social discrimination paradigm.

α-FMH, a kind gift of Dr. Carruthers Janssen Research & Development (USA) was dissolved in 0.9% saline and injected immediately after the habituation period; VUF16839, a kind gift of Prof. Leurs of Vrije Universiteit, Amsterdam (NL) was dissolved in 0.9% saline and administered 30 minutes after training or test or immediately after training phase depending the memory phase we want to study; Ciproxifan (Tocris) were dissolved in physiological saline and injected 30 minutes before training Donepezil (Sigma-Aldrich) and administered 45minutes before training was dissolved in physiological saline.

Surgery and i.c.v. infusion procedure: For α-FMH infusion, mice were anesthetised with zoletil and xylazine (45mg/kg+7,5mg/kg) and placed on a stereotaxic frame (Kopf Instruments). A stainless steel cannula (7 mm in length, OD 0.5 mm and ID 0.25 mm) was implanted in the lateral ventricle and fixed to the skull using dental cement. The following coordinates (in mm) were used according to the mouse brain atlas (Paxinos and Franklin 2007): AP -0.3; L -1; DV -1. Animals were left to recover for 7 days. In the experiment day a stainless steel injection micro-needle (2.4 mm length; OD 0.25 mm) was connected through a polyethylene catheter to a 1ml Hamilton precision syringe and then lowered into the lateral cerebral ventricle (dorsoventral, DV 2.4 mm from bregma). α-FMH, or saline were delivered via an infusion pump (5 µl) within 5 min. After infusion, the needle was left in place for 1 additional min.

$HDC^{+/+}$ mice were randomly assigned to the different experimental groups: $HDC^{+/+}$, $HDC^{+/+}$ and α-FMH or saline (i.c.v.), $HDC^{+/+}$ and VUF16839 (5mg/kg) or saline (i.p.), tested 1 hour or 24 hours after training; $HDC^{+/+}$ injected with α-FMH or saline i.c.v.), VUF16839 or saline (i.p.) tested 1 hour after training; $HDC^{+/+}$ and Ciproxifan (3mg/kg) or saline i.p. test 48 hours after training.

$HDC^{-/-}$ mice were randomly assigned to this different experimental groups: $HDC^{-/-}$ tested 1 hour or 24 hours after training, $HDC^{-/-}$ treated with VUF16839 or saline i.p tested 1 hour after training.

Social recognition paradigm: This task relies on the animal's innate tendency to explore a novel social stimulus with respect to a familiar, previously encountered congener (Okuyama 2018). The social recognition task consisted of 3 phases: habituation, training or sociability phase (T1) and a testing phase (T2). Test and stimulus mice were brought to the testing room in their home cages and were allowed to sit undisturbed in the testing room for at least 1 hour before the start of behavioural testing. Stimulus mice were placed 30 minutes under the cylinder

in the absence of the test mouse to get used to the experimental conditions. In the habituation period the mice were placed for 10 minutes in a transparent polyvinylchloride box (46 × 20 cm and 20 cm high) with two identical empty perforated cylinders (8 × 8 cm and 12cm high) in two opposite side. The arena is illuminated by a 75-W lamp suspended 50 cm above the box. 24 hours after habituation, mice were placed for 10 min into the test arena facing the same direction and in the same position in the presence of a juvenile mouse under one of the cylinders. The behaviour of mice was videotaped, and the time spent actively exploring the juvenile mouse or the empty cylinder was measured. Exploration was defined as sniffing or touching the cylinders with the nose and/or forepaws. Test section was performed 1 (Short-term memory), 24 or 48 (Long-term Memory) hours after training, during which, each mouse was placed again in the test arena for 10 min in the presence of the familiar juvenile mouse under a cylinder and a novel juvenile mouse under the other cylinder. The position of the familiar mouse (left/right) was randomized to prevent bias due to place preference. The behavior of mice during T2 was videotaped, and the exploration time of the familiar (F) and the new juvenile mice (N) were measured by a trained observer who was unaware of the treatment and genotype.

Statistical analysis: All values are expressed as means ± SEM, and the number of mice used in each experiment is also indicated. The presence of significant treatment effects was determined by a 2-way ANOVA followed by Bonferroni MCT test, as appropriate. The level of significance was set at $P \leq 0.05$. Statistical analysis was performed using GraphPad Software. The data of the social recognition test are expressed as percentage of time spent exploring the juvenile mouse and the empty cylinder during the training section ad percentage of time spent exploring the novel and familiar juvenile mice during the test phase. We also express the results as sociability index for the training and discrimination index for the test section using the equations below.

$$Sociability\ Index = \frac{(Time\ spent\ exploring\ social\ target - Time\ spent\ exploring\ non\ social\ stimulus)}{Total\ exploration\ time}$$

$$Discrimination\ Index = \frac{(Time\ spent\ exploring\ novel\ mouse - Time\ spent\ exploring\ familiar\ mouse)}{Total\ exploration\ time}$$

Results

Chronic histamine deprivation impairs long- but not short-term social recognition memory.

In this first experimental set, we analyzed the performance of $HDC^{+/+}$ and $HDC^{-/-}$ mice in social recognition test at two different time point: 1 hour and 24 hours after training.

First, we evaluated the short-term memory performing the test phase 1 hour after training. Figure 32C shows the results of the social recognition test. Mice of both genotypes remembered the social stimulus presented in the training phase because they spent significantly more time exploring the novel social stimulus. (Two-way ANOVA e Bonferroni MCT ($F_{(interaction)1,52}$=0,6295; $F_{(cylinder)1,52}$=37,25; $F_{(genotype)1,52}$=1,257e-013).

Therefore, we deduce that histamine deprivation has no influence on short-term memory consolidation. This conclusion is also supported by the discrimination index as there were no differences between genotypes.

In the second experimental set we studied the impact of chronic histamine depletion on long-term social memory. The protocol used was similar to the previous one except for the time elapsed between the acquisition sessions and the retention test which in this case is 24 hours. During the test section, 24h after training, Two-way ANOVA revealed statistically significant differences between groups (Two-way ANOVA, $F_{(interaction)1,30}$= 20,99; $F_{(cylinder)1,30}$= 11,58; $F_{(genotype)1,30}$= -3,168e-014). As shown in Figure 31C $HDC^{+/+}$ animals spent more time exploring the novel social stimulus compared to the familiar one ($p<0.001$). On the contrary, $HDC^{-/-}$ did not discriminate between the two juvenile mice. To confirm this result, we observed also a statistically significant difference in discrimination index between $HDC^{+/+}$ and $HDC^{-/-}$ mice (Unpaired t-test $p<0.01$).

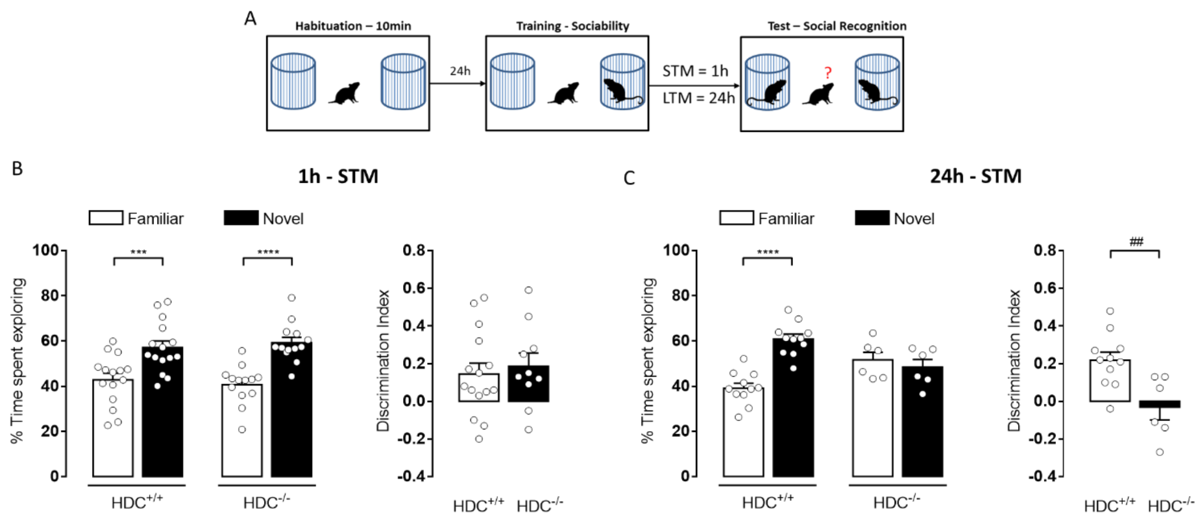


Figure 32. Impact of chronic histamine depletion on short- and long-term social recognition memory. (A) schematic representation of the experimental protocol used in normal (HDC+/+) and chronically histamine-depleted (HDC-/-) animals. (B) Performance of the animals during the test phase 1 hour after training. (C) Performance of the animals on long-term memory. The results are calculated as a percentage of time spent exploring the cages containing the different stimuli (familiar vs. novel) or mediating the discrimination index. (two-way ANOVA and Bonferroni's MCT; **** p<0.0001; ***p<0.001;Unpaired t-test ##p<0.01; n=15-6)STM: short-term memory, LTM: Long-term memory.

Acute histamine deprivation impairs long- but not short-term social recognition memory

Figure 33 show the performance of $HDC^{+/+}$ mice injected with α-FMH or saline i.c.v. in the social recognition test at two different time point: 1 hour and 24 hours after training.

To evaluate short-term memory, we performed the test phase 1 hour after training. The results of the recognition test of STM are shown in Figure 33B. Normal mice or mice pharmacologically deprived of histamine spent more time exploring the novel mouse. (Two-way ANOVA e Bonferroni MCT, $F_{(interaction)1,46}$=0.4276; $F_{(cylinders)1,46}$=26,23; $F_{(treatment)\ 1,46}$=-2,950e-014). Therefore, we suggest that acute histamine deprivation as well has no influence on short-term memory consolidation. This conclusion is also supported by the discrimination index because there were no differences between genotypes.

In Figure 33C the second part of this experiment is shown. We studied the effect of an acute depletion of central histaminergic levels on long-term memory tested 24 hours after training.

As show in Figure 33C $HDC^{+/+}$ mice injected with vehicle spent more time exploring the novel social stimulus compared to the familiar one (****p<0.0001). On the contrary, mice injected with α-FMH did not discriminate between the two juvenile mice (Two-way ANOVA e Bonferroni MCT, $F_{(interaction)1,36}$=22,58; $F_{(cylinders)1,36}$=15,60; $F_{(treatment)\ 1,36}$=-4,426e-014). To confirm this result, we observed also a statistically significant difference in discrimination index between mice injected with α-FMH and with vehicle (Unpaired t-test ###p<0.001).

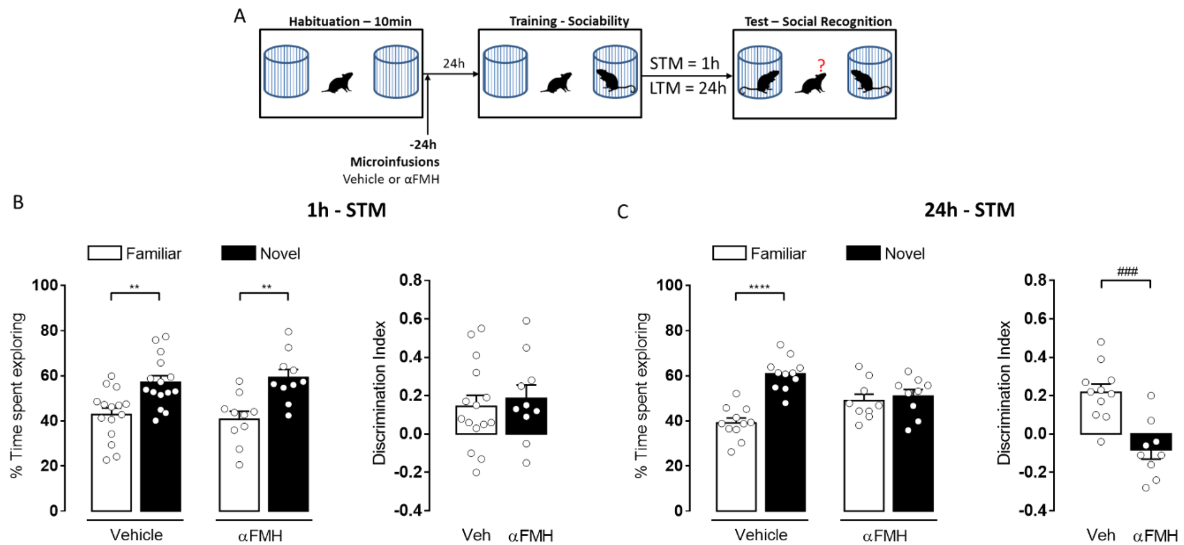


Figure 33. Evaluation of short- and long-term memory in the Social recognition test of acutely histamine-depleted animals ($HDC^{+/+}$ injected with α-FMH). (A) Schematic representation of the experimental protocol; (B) Results of performance in short-term memory; (C) Performance of the animals in the test section performed 24 hour after the training. Results are expressed as a percentage of exploration time and through the discrimination index. (Two-way ANOVA and Bonferroni MCT, ****p<0.0001; **p<0.01; Unpaired t-test $^{###}$p<0.001; n=9-15).

H_3R agonism impairs both short and long-term social recognition memory

In the next sets of experiment, we studied the performance of $HDC^{+/+}$ mice treated with an H_3R agonist, VUF 16839 (5mg/kg) injected 30 minutes before training section in the social recognition test performed 1 or 24 hours after training.

In Figure 34 the results of $HDC^{+/+}$ mice treated with VUF16839 or vehicle i.p. in short and long-term social recognition memory are shown.

First of all, we performed the test 1 hour after the training phase to evaluate the short-term social recognition memory. Two-way ANOVA revealed a statistically significant difference between groups because $HDC^{+/+}$ mice treated with vehicle spent more time exploring the novel juvenile mouse compared to the familiar one, but the animals treated with VUF16839 did not discriminate between the two juveniles indicating a short-term memory impairment (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)1,46}$=10,50; $F_{(cylinders)1,46}$=1,519; $F_{(treatment)\ 1,46}$=-4,547e-014;**p<0.01).

In the discrimination index we observed a statistically significant difference between the two groups indicating a short-term memory impairment of the animals treated with VUF16839 (Unpaired t-test, $^{#}$p<0.05) involving neurotransmitters other than histamine.

Figure 34C show the results obtained in the long-term social memory experimental set. The test section was performed 24 hours after training. The results show that $HDC^{+/+}$ mice treated with vehicle spent more time exploring the novel stimulus compared to the familiar one (****$p<0.0001$). On the contrary, mice treated with VUF16839 spent equal time exploring the familiar and the novel juvenile mice, so they show a long-term memory impairment (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)1,36}=18{,}60$; $F_{(cylinders)1,36}=17{,}80$; $F_{(treatment)\ 1,36}=2{,}127e\text{-}014$). To confirm this result, we observed also a statistically significant difference in discrimination index between mice injected with α-FMH and with vehicle (Unpaired t-test ##$p<0.01$).

In this case we observed that the reduction of histamine levels mediated the injection of H_3 receptor agonist impaired both short and long-term memory on the contrary to chronic or acute histamine deprivation. This may be because the H_3 receptor is also a heteroreceptor and therefore can impact the release of other neurotransmitters (Haas et al. 2008).

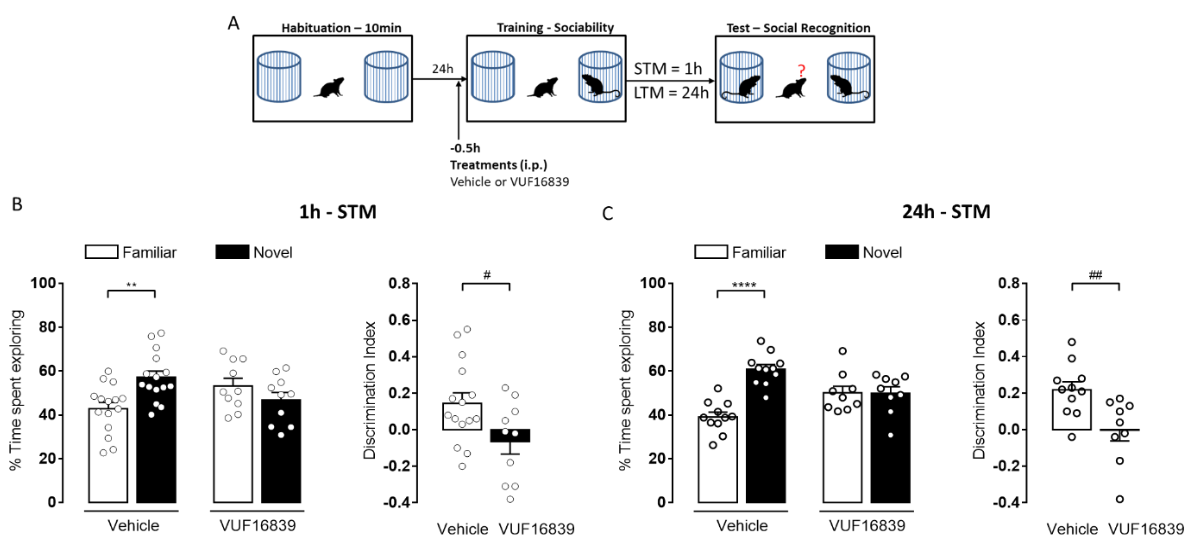


Figure 34. Effects of VUF16839 treatment in $HDC^{+/+}$ mice on sociability and short- and long-term social recognition memory. (A) schematic representation of the experimental protocol used in $HDC^{+/+}$ treated with VUF16839 and vehicle. The results are calculated as a percentage of time spent exploring the cages containing the different stimuli (familiar vs. novel) or mediating the discrimination index. (B) Performance of the animals 1 hour after training (STM). (C) Performance of the animals on the test phase performed 24h after training (LTM) (Two-way ANOVA and Bonferroni's MCT; ****$P<0.0001$; **$p<0.01$; Unpaired t-test, ##$p<0.01$; #$p<0.001$; n9-15).

H_3R agonist-induced amnesic is not related with histaminergic neurotransmission.

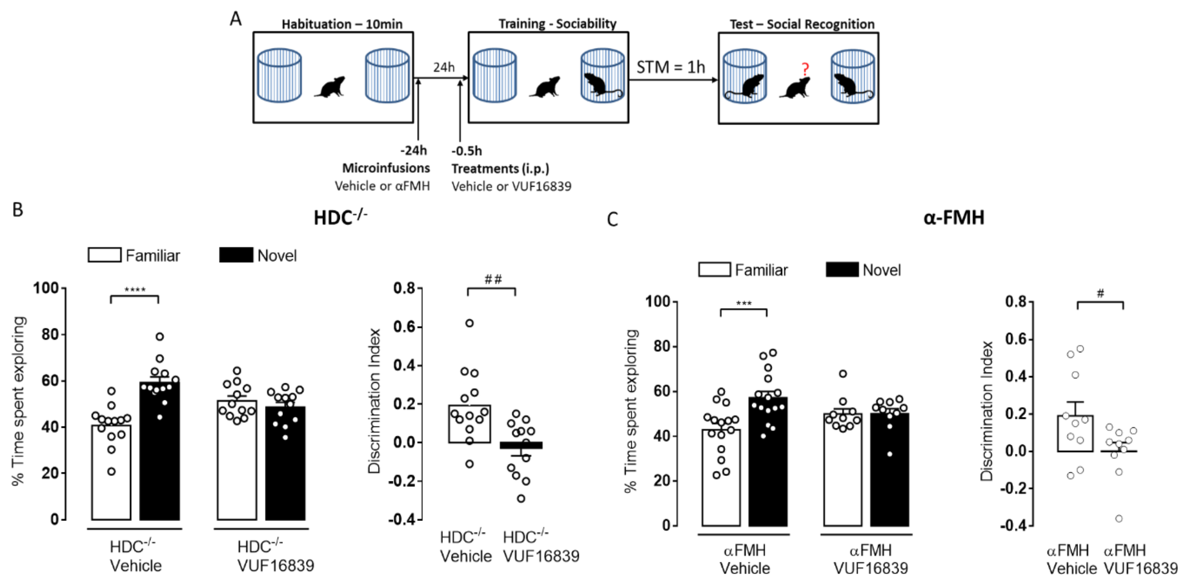
Given the results obtained with the administration of the H_3R agonist, in this experimental set we evaluated whether the effect of VUF16839 was related to the histaminergic system or not. For this reason, we administered VUF16839 to chronically ($HDC^{-/-}$) or acutely (α-FMH i.c.v.) histamine-depleted animals 30 minutes before training and we test these mice in the social recognition for the short-term memory.

Figure 35 show the results of histamine depleted mice treated with VUF16839 or vehicle i.p. in short-term social recognition memory. As previously observe $HDC^{-/-}$ didn't have a short-term memory impairment and also in this casa two-way ANOVA revealed a statistically significant difference between groups because $HDC^{-/-}$ mice treated with vehicle spent more time exploring the novel juvenile mouse compared to the familiar one, but when treated with VUF16839 this animals chronically lacking of histamine did not recognize the familiar juvenile and spent the same time exploring the two juvenile mice (Two-way ANOVA and Bonferroni MCT; $F_{(interaction)1,46}$=22,68; $F_{(cylinders)1,46}$=12,42; $F_{(treatment)\ 1,46}$=-7,230e-014).

Also, in the discrimination index we observed a statistically significant difference between the two groups indicating a short-term memory impairment of the $HDC^{-/-}$ animals treated with VUF16839 (Unpaired t-test, $^{\#\#}p<0.01$) involving neurotransmitters other than histamine.

Figure 35C show the results obtained with $HDC^{+/+}$ mice injected with the suicide inhibitor of HDC enzyme, α-FMH in order to obtain an acute depletion of histamine levels. These mice were tested in the social recognition memory task 1 hour after training. The results show that, also in this case, $HDC^{+/+}$ mice injected with α-FMH i.c.v. and treated with vehicle spent more time exploring the novel stimulus compared to the familiar one (***$p<0.001$). On the contrary, mice treated with VUF16839 spent equal time exploring the familiar and the novel juvenile mice, so they show a short-term memory impairment (Two-way ANOVA and Bonferroni MCT; CAMBIA NUMERI $F_{(interaction)1,36}$=9,248; $F_{(cylinders)1,36}$=9,405; $F_{(treatment)\ 1,36}$=0,0). To confirm this result, we observed also a statistically significant difference in discrimination index between mice injected with α-FMH and with vehicle (Unpaired t-test $^{\#}p<0.05$).

So, we observed that the effect of the H_3R agonist is not mediated by the histaminergic system but by other neurotransmitters.



<u>Figure 35.</u> Impact of H3R agonist VUF16839 in chronic and acute histamine depletion on short-term social recognition memory. (A) schematic representation of the experimental protocol used in chronically depleted ($HDC^{-/-}$) mice and normal ($HDC^{+/+}$) animals injected with α-FMH i.c.v. (B) Performance of $HDC^{-/-}$ mice. (C) Performance of normal ($HDC^{+/+}$) animals injected with α-FMH i.c.v.. The results are calculated as a percentage of time spent exploring the cages containing the different stimuli (familiar vs. novel) or mediating the discrimination index. (two-way ANOVA and Bonferroni's MCT; ****P<0.0001; ***p<0.001; Unpaired t-test; $^{\#\#}$p<0.01; $^{\#}$p<0.05; n=10-15)

Donepezil prevents H3R agonist-induced memory impairment.

Acetylcholinesterase inhibitors such as donepezil, are currently used for the relief of the cognitive deficits associated with mild to moderate Alzheimer's disease (Micheau and Marighetto 2011), Histaminergic neurotransmission affects the ACh release and the interplay between the two systems also affects memory formation (Blandina et al. 2004).

Figure 36 shows the effect of donepezil (3mg / kg) administered 45 minutes before training in animals subsequently treated with the H3R agonist, VUF16839 (5mg / kg) 30 minutes before training.

We evaluate short-term memory 1h after training. The results of the recognition test demonstrated that mice treated with VUF16839, as noted above, show memory impairment. But mice previously treated with Donepezil spend more time exploring the novel juvenile than the familiar one (Two-way ANOVA e Bonferroni MCT, $F_{(interaction)1,36}$=23,58; $F_{(cylinders)1,36}$=7,162; $F_{(treatment)\ 1,36}$=-0,0) indicating that Acetylcholinesterase inhibitors prevents the VUF16839 memory impairment.

The results are also supported by the observed differences between animals treated with donepezil or vehicle in the discrimination index (Unpaired t-test $^{\#\#}$p<0.01).

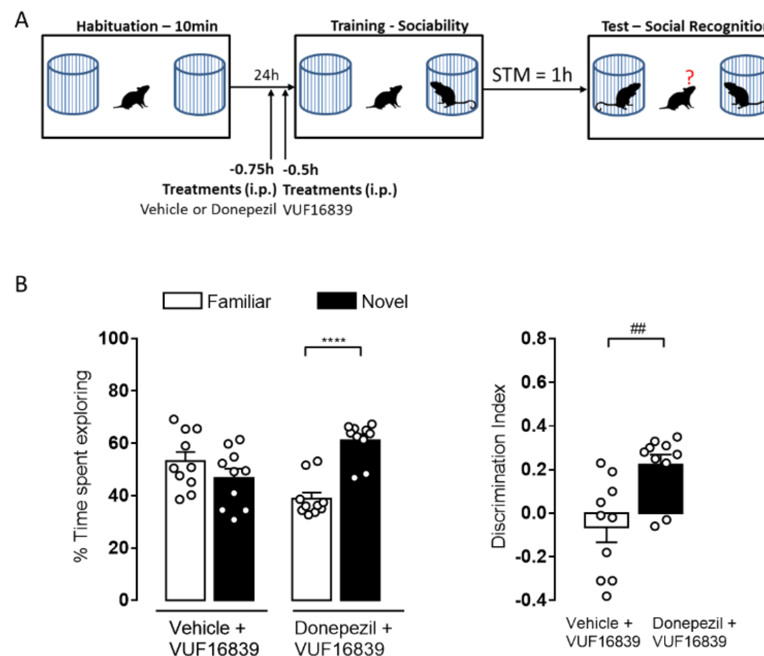


Figure 36. Evaluation of the effect of the acetylcholinesterase inhibitor Donepezil on animals treated with VUF16839 in short-term memory. (A) Schematic representation of the experimental protocol; (B) Results of performance in short-term memory. Results are expressed as a percentage of exploration time and through the discrimination index. (Two-way ANOVA and Bonferroni MCT, ****p<0.0001, Unpaired t-test; ##p<0.01; n=10).

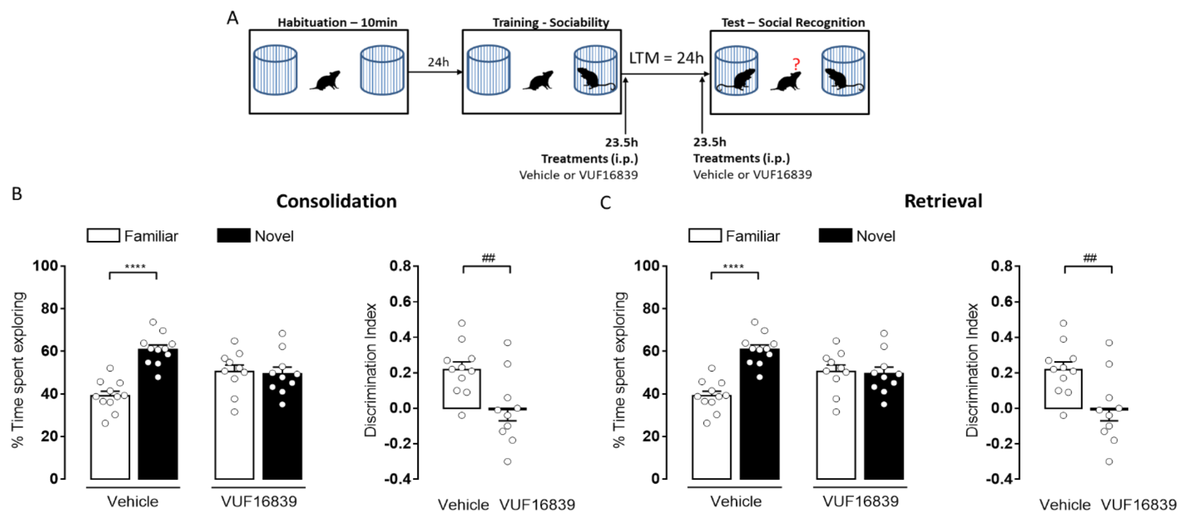
H_3R agonism impairs acquisition, consolidation and retrieval of long-term social recognition memory.

As we observed in Figure 34C, the administration of VUF16839 30 minutes before training impaired the acquisition of long-term memory. For this reason, in this experimental set we evaluated whether the H_3R agonism could also influence other phases of mnemonic process.

So, in Figure 37 is shown the effect of VUF16839 or vehicle injection immediately after training to evaluate memory consolidation (Figure 37B), or 30 minutes before LTM test section to evaluate memory retrieval (Figure 37C).

Two-way ANOVA revealed statistical differences in the $HDC^{+/+}$ mice treated with vehicle immediately after training (Two-way ANOVA e Bonferroni MCT, $F_{(interaction)1,38}$=18,46; $F_{(cylinders)1,38}$=15,41; $F_{(treatment)\ 1,38}$=-1,245e-13) or 30 minutes before test (Two-way ANOVA e Bonferroni MCT, $F_{(interaction)1,37}$=56,56; $F_{(cylinders)1,37}$=422,6; $F_{(treatment)\ 1,37}$=0,03392), but revealed no differences in the animals treated with VUF16839 indicating that this H_3R agonist impairs also consolidation and retrieval of memory.

This difference between the two treatments is also demonstrated by discrimination index (Unpaired t-test $^{\#\#}p<0.01$)



<u>Figure 37</u>. Effect of VUF16839 on consolidation and retrieval of long-term memory. (A) Schematic representation of the experimental protocol; (B) Results of H_3R agonist administered immediately after training to evaluated consolidation phase; (C) Effect of VUF16839 when administered 30 minutes before test to evaluated retrieval. Results are expressed as a percentage of exploration time and through the discrimination index. (Two-way ANOVA and Bonferroni MCT, ****p<0.0001, Unpaired t-test; $^{\#\#}p<0.01$ ***p<0.001; n=9-11).

Increased histamine release is responsible for H_3R antagonist-induced procognitive effect.

In this final experiment, we analyzed the performance of normal and histamine depleted ($HDC^{-/-}$ or $HDC^{+/+}$ injected with α-FMH i.c.v.) mice treated with Ciproxifan (3mg/kg), an H_3 receptor antagonist, or vehicle in social recognition performed 48 hours after training.

We performed the recognition test 48 hours after training, to assess whether ciproxifan could ameliorate a physiologically decaying memory.

Figure 38B shows the results of the social recognition test of $HDC^{+/+}$ and $HDC^{-/-}$ mice. Two-way ANOVA analysis show statistical difference between groups (Two-way ANOVA and Bonferroni MCT, $F_{(interaction)3,66}$=11,30; $F_{(cylinder)1,66}$=26,26; $F_{(treatments)3,66}$= 4,203e-014) but in this case $HDC^{+/+}$ mice treated with vehicle, did not discriminate between the two juvenile mice as a result of normal, time-dependent forgetting. On the contrary, we observe that $HDC^{+/+}$ mice treated with Ciproxifan (3mg/Kg) spent more time exploring the novel mouse compared to the familiar one (****p<0.0001) indicating that increased histamine levels produce an amelioration in social memory consolidation, but $HDC^{-/-}$ mice treated with Ciproxifan didn't discriminate between the two juveniles demonstrating that this effect is mediated by the central histaminergic

system. This conclusion is also supported by the discrimination index as there is a statistically significant difference between animals treated with Ciproxifan or with vehicle (One-way ANOVA and Bonferroni MCT, $F_{(interaction)3,34}$=4,998) in $HDC^{+/+}$ animals.

To confirm these results, we use also the $HDC^{+/+}$ mice injected with α-FMH to observe the effects of an acute depletion of histamine levels and we can observe the same results seen in the genetic model of histamine deficiency. Two-way ANOVA demonstrated that $HDC^{+/+}$ injected with vehicle i.c.v. and treated with Ciproxifan spent more time exploring the novel juvenile mouse (Two-way ANOVA and Bonferroni MCT, $F_{(interaction)3,58}$=32,35; $F_{(cylinder)1,58}$=10,18; $F_{(treatments)3,58}$= 1,223e-013), but also in this case the absence of histamine avoids the effect of H_3R antagonist. To support these results, also in this case, we calculated the discrimination index which showed differences between the groups of animals injected with vehicle i.c.v. and treated with ciproxifan or vehicle (One-way ANOVA and Bonferroni MCT, $F_{(interaction)3,33}$=14,63) (Figure 38C).

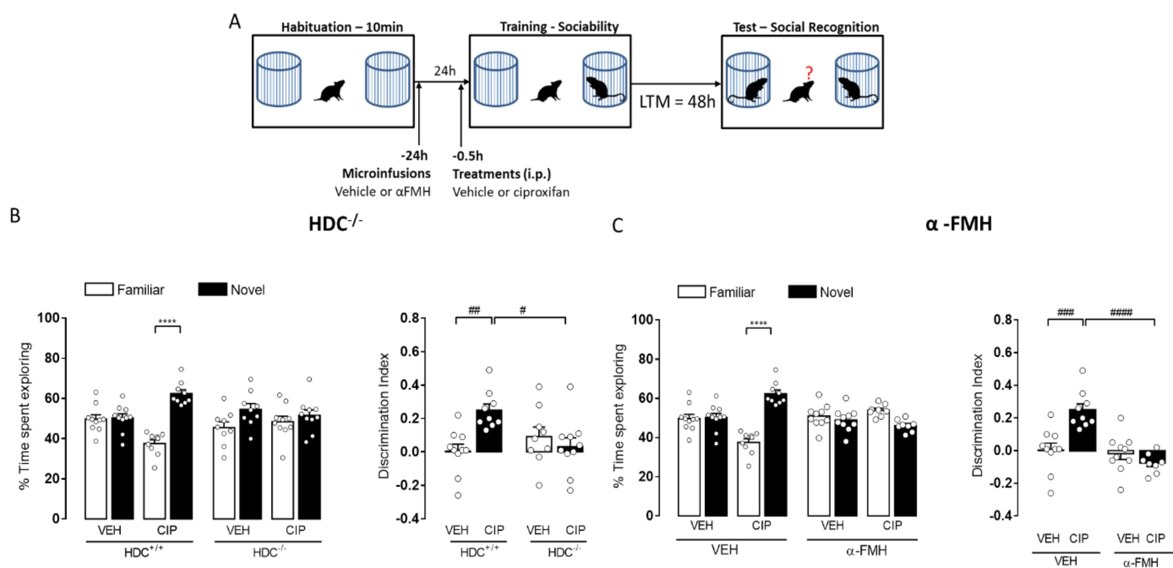


Figure 38. Evaluation of long-term memory in the Social recognition test of normal and histamine depleted mice treated with Ciproxifan (3mg/kg) or vehicle. (A) Schematic representation of the experimental protocol; (B) Results of $HDC^{+/+}$ or $HDC^{-/-}$ animals in social recognition performed 48 hours after training; (C) Performance of $HDC^{+/+}$ mice injected with α-FMH or vehicle and treated with Ciproxifan or vehicle. Results are expressed as a percentage of exploration time and through the sociability or discrimination index. (Two-way ANOVA and Bonferroni MCT, ****p<0.0001, One-way ANOVA and Bonferroni MCT, $^{####}$p<0.0001; $^{###}$p<0.001; $^{##}$p<0.01; n8-10).

Treatments do not affect mice sociability

During the training phase of each experimental set, we evaluated the levels of sociability evaluating the time that each animal spends exploring the juvenile mouse placed under a cylinder compared to the time spent exploring another identical empty cylinder. The results are shown in table 9 and show that no treatment negatively affects sociability as all animals spent more time exploring the juvenile mouse compared to the inanimate object. The results are also expressed through the Sociability index.

Table 9. Effects of different treatments on sociability. Sociability is express as percentage of time spent exploring the social stimulus or the non social one and as Sociability index. All the results are reported as mean ±SD.

TIME SPENT EXPLORING (Mean ± SD)							
	Genotype	**i.c.v.**	**i.p**	**Inter trial interval (h)**	**Social: Time spent exploring (%)**	**Non Social: Time spent exploring (%)**	**Sociability index**
Experiment 1	$HDC^{+/+}$	-	-	1	80,30 ± 8,22	19,70 ± 8,22	0,60 ± 0,067
	$HDC^{-/-}$	-	-	1	68,56 ± 9,54	31,44 ± 9,54	0,37 ± 0,0,78
	$HDC^{+/+}$	-	-	24	81,85 ± 6,85	18,14 ± 6,85	0,48 ± 0,0,62
	$HDC^{-/-}$	-	-	24	68,6 ± 4,84	31,40 ± 4,84	0,37 ± 0,10
Experiment 2	$HDC^{+/+}$	VEH	-	1	65,70 ± 4,24	34,29 ±4,24	0,31 ± 0,085
	$HDC^{+/+}$	α-FMH	-	1	76,29 ± 3,17	23,71 ± 3,17	0,53 ± 0,063
	$HDC^{+/+}$	VEH	-	24	83,42 ± 7,92	16,46 ± 7,92	0,52 ± 0,059
	$HDC^{+/+}$	α-FMH	-	24	72,32 ± 10,67	27,68 ± 10,67	0,64 ± 0,19
Experiment 3	$HDC^{+/+}$	-	VEH	1	64,76 ± 9,27	36,29 ± 9,27	0,35± 0,054
	$HDC^{+/+}$	-	VUF16839	1	81,88 ± 7,24	18,12 ± 7,24	0,64 ± 0,12
	$HDC^{+/+}$	-	VEH	24	84,75 ± 6,37	15,25 ± 6,37	0,44 ± 0,068
	$HDC^{+/+}$	-	VUF16839	24	59,25 ± 7,53	40,75 ± 7,53	0,18 ± 0,15
Experiment 4	$HDC^{-/-}$	-	VEH	1	66,99 ± 7,42	33,01 ± 7,42	0,34 ± 0,053
	$HDC^{-/-}$	-	VUF16839	1	69,74 ± 7,58	30,25 ± 7,58	0,39 ± 0,048
	$HDC^{+/+}$	α-FMH	VEH	1	70,38 ± 9,90	29,62 ± 9,90	0,41 ± 0,25
	$HDC^{+/+}$	α-FMH	VUF16839	1	66,90 ± 12,43	33,10 ± 12,43	0,34 ± 0,16
Experiment 5	$HDC^{+/+}$	-	VEH + VUF16839	1	81,88 ± 7,24	18,12 ± 7,24	0,64 ± 0,12
	HDC+/+	-	DONEPEZIL +VUF	1	69,45 ± 6,95	30,55 ± 6,95	0,37 ± 0,12
Experiment 6	$HDC^{+/+}$	-	VEH	24 (Consolidation)	82,14 ± 7,17	17,85 ± 7,17	0,49 ± 0,066
	$HDC^{+/+}$	-	VUF16839	24 (Consolidation)	65,68 ± 8,82	34,32 ± 8,82	0,31 ± 0,056
	$HDC^{+/+}$	-	VEH	24 (Retrieval)	86,29 ± 7,27	13,71 ± 7,27	0,49 ± 0,17
	$HDC^{+/+}$	-	VUF16839	24 (Retrieval)	65,60 ± 7,93	34,40 ± 7,93	0,31 ± 0,17
Experiment 7	$HDC^{+/+}$	-	VEH	48	71,21 ± 6,47	28,78 ± 6,47	0,43 ± 0,14
	$HDC^{+/+}$	-	CIPROXIFAN	48	63,59 ± 10,25	36,41 ± 10,25	0,12 ± 0,064
	$HDC^{-/-}$	-	VEH	48	62,76 ± 12,19	37,24 ± 12,19	0,26 ± 0,24
	$HDC^{-/-}$	-	CIPROXIFAN	48	76,23 ± 5,07	23,77 ± 5,07	0,52 ± 0,10
	$HDC^{+/+}$	VEH	VEH	48	71,84 ± 2,79	28,16 ± 2,79	0,49 ± 0,19
	$HDC^{+/+}$	VEH	CIPROXIFAN	48	64,32 ± 2,72	35,68 ± 2,72	0,12 ± 0,065
	$HDC^{+/+}$	α-FMH	VEH	48	69,47 ± 6,51	30,53 ± 6,51	0,39 ± 0,13
	$HDC^{+/+}$	α-FMH	CIPROXIFAN	48	70,70 ± 8,66	29,30 ± 8,66	0,41 ± 0,17

Treatments do not affect general motor activity

General motor activity is evaluated indirectly by evaluating the total time that the animals spend exploring the two cylinders during the test phase. As we can see in table 10, no treatment produces statistically significant differences in the total exploration time thus indicating that there are no differences in the motor activity of mice belonging to different genotypes or treatments group.

<u>Table 10</u>. Effects of different treatments in locomotion. Motor activity is express as mean ±SD of the time spent in seconds (s) exploring the two cylinders during the test phase.

TIME SPENT EXPLORING (TOTAL) Mean ± SD						
	Genotype	**i.c.v.**	**i.p**	**Inter trial interval (h)**	**Motor activity (s)**	**F**
Experiment 1	$HDC^{+/+}$	-	-	1	137,50 ± 39,55	1,346
	$HDC^{-/-}$	-	-	1	134,77 ± 34,09	
	$HDC^{+/+}$	-	-	24	149,69 ± 64,50	4,981
	$HDC^{-/-}$	-	-	24	150,62 ± 28,90	
Experiment 2	$HDC^{+/+}$	VEH	-	1	130,47 ± 45,30	2,094
	$HDC^{+/+}$	α-FMH	-	1	138,91 ± 65,54	
	$HDC^{+/+}$	VEH	-	24	149,69 ± 64,50	1,097
	$HDC^{+/+}$	α-FMH	-	24	127,87 ± 61,59	
Experiment 3	$HDC^{+/+}$	-	VEH	1	135,65 ± 44,83	2,396
	$HDC^{+/+}$	-	VUF16839	1	148,27 ± 69,40	
	$HDC^{+/+}$	-	VEH	24	149,69 ± 64,50	1,038
	$HDC^{+/+}$	-	VUF16839	24	122,2 ± 63,3	
Experiment 4	$HDC^{-/-}$	-	VEH	1	134,77 ± 34,09	1,105
	$HDC^{-/-}$	-	VUF16839	1	91,78 ± 32,44	
	$HDC^{+/+}$	α-FMH	VEH	1	138,91 ± 65,54	2,699
	$HDC^{+/+}$	α-FMH	VUF16839	1	88,46 ± 39,90	
Experiment 5	$HDC^{+/+}$	-	VEH + VUF16839	1	148,27 ± 69,40	3,314
	HDC+/+	-	DONEPEZIL +VUF	1	119,36 ± 38,12	
Experiment 6	$HDC^{+/+}$	-	VEH	24 (Consolidation)	66,61 ± 22,33	1,27
	$HDC^{+/+}$	-	VUF16839	24 (Consolidation)	86,71 ± 19,81	
	$HDC^{+/+}$	-	VEH	24 (Retrieval)	73,29 ± 21,45	3,944
	$HDC^{+/+}$	-	VUF16839	24 (Retrieval)	102,57 ± 42,60	
Experiment 7	$HDC^{+/+}$	-	VEH	48	111,42 ± 23,04	1,633
	$HDC^{+/+}$	-	CIPROXIFAN	48	95,03 ± 18,02	
	$HDC^{-/-}$	-	VEH	48	93,33 ± 42,28	1,362
	$HDC^{-/-}$	-	CIPROXIFAN	48	101,5 ± 36,22	
	$HDC^{+/+}$	VEH	VEH	48	111,42 ± 23,04	1,633
	$HDC^{+/+}$	VEH	CIPROXIFAN	48	95,03 ± 18,02	
	$HDC^{+/+}$	α-FMH	VEH	48	151,56 ± 84,60	5,184
	$HDC^{+/+}$	α-FMH	CIPROXIFAN	48	154,68 ± 37,16	

Conclusion: part III

Histaminergic pharmacology and in particular the effects of H_3 receptors are very complex. H_3 receptors act as both auto and hetero-receptors and therefore they modulate several downstream systems in the brain. Histamine acting in different brain sites, has an important role as a regulator of memory consolidation/retrieval in various learning paradigms. The role of histamine receptors in recognition memory has been extensively studied using both specific ligands and also transgenic animals.

Data obtained until now indicate that alterations in the histaminergic system are associated with cognitive deficits observed in many neurodegenerative diseases, suggesting histaminergic receptors as possible targets for the discovery of new drugs (Provensi et al. 2018a). Due to its actions as an auto/heteroreceptor, regulating not only histamine synthesis but also the release of other neurotransmitters critically involved in cognition, the H_3 receptor has received great attention by the scientific community as a good target for the development of new centrally acting drugs, and many academic groups as well as pharmaceutical companies have synthesized numerous selective and potent H_3 receptor ligands (Sadek et al. 2016).

This part of my thesis focused on social recognition test, a protocol used for the evaluation of memory. We studied the effects of brain histamine in sociability and in short and long-term social recognition memory. We evaluated the effect on memory of genetic or pharmacological depletion of histamine levels by using $HDC^{-/-}$ mice or $HDC^{+/+}$ mice treated with i.c.v injection of α-FMH, or i.p. injection of the H_3 receptor agonist VUF16839.

We found that the lack of acute or chronic histamine did not induce sociability deficits. $HDC^{-/-}$ mice or $HDC^{+/+}$ mice treated with α-FMH showed intact short-term memory, but long-term memory deficit. However, $HDC^{+/+}$ mice treated with VUF16839 showed short-term memory impairments as well. Hence, VUF16839 that presumably abrogates histamine synthesis and release, had an amnesic effect on both short-term and long-term memory, contrary to $HDC^{-/-}$ or α-FMH treated mice. A plausible explanation for this phenomenon may lie in the fact that the H_3 receptor is also a heteroreceptor that controls the release of other neurotransmitters (Haas et al. 2008). Presumably, its heterogeneous distribution induced a reduction of other neurotransmitters' release which are mainly involved in short-term memory.

To validate our hypothesis, we treated $HDC^{-/-}$ and α-FMH mice with VUF16839 and we observed a similar result as with the H_3 receptor agonist, i.e. impaired short-term memory. We speculate that deletion of the HDC gene in $HDC^{-/-}$ mice and α-FMH treatment are highly

selective procedures to abolish histamine and, for this reason, they induced only long-term memory impairment.
To evaluate whether the effect of VUF16839 was mediated by other neurotransmitters, we treated the animals with Donepezil, an inhibitor of the acetylcholinesterase enzyme, commonly used for the treatment of Alzheimer's disease (Micheau and Marighetto 2011). The data show that pretreatment with Donepezil prevented the negative effect of VUF16839 on social memory acquisition confirming the involvement of neurotransmitters other than histamine, specifically acetylcholine, in the effect of VUF16839.
If therefore a deficit of histamine inevitably leads to mnemonic impairment, its increase should enhance the consolidation of memory. We performed the test 48 hours after training when mice experience physiological forgetting. We treated the normal or histamine-lacking mice with a systemic administration of Ciproxifan, an H_3 receptor antagonist to confirm this hypothesis and we observed an amelioration in memory consolidation of $HDC^{+/+}$ mice, but not in that of chronically or acutely histamine-depleted animals, indicating that the effect of Ciproxifan it is mediated by the central histaminergic system and not by other neurotransmitters.

Discussion

A healthy nutritional status is essential for proper brain development and for the maintenance of optimal cognitive function during adulthood and aging. We demonstrated that a diet enriched with ω-3 polyunsaturated fatty acids (ω-3 PUFAs) and vitamin A protects against the cognitive and neurochemical consequences of chronic stress during adolescence. Furthermore, we showed that the amelioration is maintained through adulthood. In this regard, it is known that inclusion of ω-3 PUFAs in the diet can affect neurotransmission by modulating neurotransmitter reuptake and improves the cholinergic transmission in the brain, and consequently improves cognitive performances (Willis et al., 2009). Contrary to the effects of ω-3 PUFAs consumption, a diet high in saturated fats is a risk factor for various mental health problems including depression and cognitive dysfunction (Sánchez-Villegas et al., 2011).
Our data, therefore, strongly suggest that a healthy diet supplemented with ω-3 PUFAs and vitamin A prevents deleterious cognitive impairment induced by social instability stress during adolescence, and that amelioration is maintained through adulthood, suggesting that a healthy diet may have long-lasting beneficial effects and help fight off neurodegenerative diseases.
A working hypothesis in our laboratory holds that the brain histaminergic system allocates to peripheral stimuli i.e. hormones, diet-derived micronutrients or microbiota-derived products the salience necessary to unfold the appropriate behaviours.
The histaminergic system holds a key position in the regulation of basic body functions, including the sleep–wake cycle, energy and endocrine homeostasis, synaptic plasticity and learning (Haas and Panula 2003). Histamine release is a sensitive indicator of stress (Taylor and Snyder 1971a, Verdière, Rose and Schwartz 1977), and chronic restraint and/or metabolic stress are among the most potent activators of histamine neurons in the TMN (Miklós and Kovács 2003). Not surprisingly, current research is providing evidence that malfunctioning of the histaminergic system is associated with neuropathological disorders (Shan et al. 2017). There is extensive evidence that histaminergic neurons detect acute stress-induced signals. Exposure to restraint and cold increased histamine turn over in the rat hypothalamus (Taylor and Snyder 1971b); hypercapnic loading (Haxhiu et al. 2001), insulin-induced hypoglycaemia, and foot shock (Haxhiu et al. 2001, Miklós and Kovács 2003) activated histaminergic neurons in a stressor- and neuron subgroup-specific manner.
Distinct subgroups of hypothalamic histamine neurons respond to immobility, foot shock, hypoglycemia, and dehydration, suggesting a functional heterogeneity of histaminergic TMN neurons (Miklós and Kovács 2003). TMN neurons are influenced by a number of

neuroendocrine signals (Gotoh et al. 2005) and may integrate exteroceptive and interoceptive state cues in the control of stress induced arousal. Histamine mediates the stress-induced neuroendocrine hormone surges of ACTH, β-endorphin, and AVP from the pituitary (Kjaer et al. 1992) and controls stress related activity of aminergic systems, including serotonin-, norepinephrine-, dopamine-, and acetylcholine-containing neurons. As an integral part of the neural networks generating autonomic patterns (Saper 2002), histamine neurons interfere with AVP- and CRH-positive sympathetic command neurons (Krout, Mettenleiter and Loewy 2003) in the PVN and LHA (Whitcup et al. 2004) to influence sympathoadrenal outflow, cardiovascular functions, and complex stress-related behaviours such as flight-fight or grooming.

In addition, histamine can be involved in the beneficial effects of ω-3 PUFAs. In rats fed a high-saturated fat diet, a reduced H_1 receptor binding density in many brain areas was observed (Wu et al., 2013). Interestingly, the reduction of H_1 receptor binding densities in some of these areas (substantia nigra and caudate putamen) was prevented by supplementing the high-fat diet with the ω-PUFA DHA; the supplemented diet also prevented the negative effect of high-fat diet in cognitive functions. H_1 receptor expression is reduced in depressed patients, whereas ω-3 PUFAs, specifically DHA, levels in serum and red blood cells membranes are reduced in bipolar and major depression patients, with a greater deficit in bipolar disorder patients (McNamara et al., 2010). As a consequence, evidence suggests that coincident alterations in histaminergic system and lipid composition in depression could be causally linked.

Therefore, during my doctorate, I explored the relationship between the brain histaminergic system and the enriched diet or the lipid OEA on the behavioural outcomes of chronic social defeat stress, a preclinical paradigm that more closely reproduces some of the symptoms observed in depression (Menard et al., 2017). Indeed, our data demonstrate that both a diet enriched in ω-3 PUFA and Vitamin A as well as the administration of a gut hormone such as OEA reduces social avoidance induced by stress and improves the cognitive performance of stressed mice. However, these effects are lost in mice that do not synthesise histamine, strengthening our hypothesis that peripheral signals generated by both the enriched diet and OEA converge onto the central histaminergic system that in turn provides the necessary central signalling to prevent stress-induced cognitive deficits and social aversion.

Most CNS functions may be modulated so as to be activated or deactivated, accelerated or slowed down, and enhanced or diminished, but the exact course of action is determined by the needs relevant for a particular moment (Izquierdo and Medina 1997). Memory is no exception

to this rule and can thus be modulated by experiences occurring about the time when it is learned, consolidated, or retrieved (Cahill, McGaugh and Weinberger 2001). The major modulatory systems are composed of diffusely spread fibres bundles that reach a broad area in the CNS. These fibres originate from nuclei in the brainstem, diencephalon, and basal forebrain. They act by means of several neurotransmitters, including acetylcholine, noradrenaline, dopamine, serotonin, and histamine (Cahill and McGaugh 1998, Izquierdo and McGaugh 2000, Brown, Stevens and Haas 2001).

Histamine is known to decrease calcium-dependent membrane conductance in the hippocampus, to increase neuronal excitability (Selbach et al. 1997), and control high-frequency oscillations (Knoche et al. 2003, Ponomarenko et al. 2003), and it also facilitates NMDA glutamatergic receptor mediated responses (Bekkers 1993). However, the part histaminergic circuits play in mnemonic systems is complex. Histamine seems to have different effects in distinct brain regions and may have modulatory effects that differ according to memory type. Various preclinical studies using KO mice have shown that histaminergic dysfunction induces learning and memory impairment (Dere et al. 2010).

All four histamine receptors are expressed in the brain, but the H_3 receptor became the most promising drug target for the treatment of neuropathic pain, sleep–wake disorders and cognitive impairment associated with ADHD, schizophrenia, AD and PD (Passani and Blandina 2011).

Consistently, systemic treatment with H_3 antagonists, known to increase synaptic levels of endogenous histamine by blocking inhibitory histamine autoreceptors (Arrang, Garbarg and Schwartz 1983), enhanced the performance of rat pups in a multi-trial, inhibitory avoidance response, a task modelling aspects of ADHD and other disorders in which vigilance, impulsivity and/or cognitive performance are impaired (Fox et al. 2002, Komater et al. 2003).

Early work by the group of Blandina et al. demonstrated that systemic administration of the H_3 agonists Imetit or R-α-methylhistamine prior to the acquisition session in the object discrimination test impaired short-term (1h) memory (Blandina et al. 1996). Furthermore, scopolamine-induced memory impairment was prevented by pretreatment with the H_3 receptor antagonists thioperamide or Clobenpropit (Giovannini et al. 1999). Our research group recently demonstrated that administration of the non-imidazole H_3 receptor antagonist, ABT-239, to wild-type mice before training and retention test improved memory in the object recognition paradigm (Provensi et al. 2016b).

Our results reported in this thesis are in line with these findings because they show that agonism or antagonism of H_3 receptors can modulate also the social recognition memory. I observed that H_3 receptor agonist (VUF16839) induced social recognition memory impairment when tested

both for short and long-term memory; as discuss before, this implies the involvement of other neurotransmitters including acetylcholine. On the contrary, the H_3 receptor antagonist (Ciproxifan) improved social memory in wild type, but not in $HDC^{-/-}$ mice suggesting that endogenous histamine is crucial for the mnemonic effects of these H_3 receptor ligands.

A wide variety of studies agree that the neuronal histaminergic system regulates some forms of cognition, and, inevitably, reports that pharmacological blockade of central H_3 receptors exerted procognitive activity in several cognitive tasks has raised considerable interest.

Recent data indicate that alterations in several components of the histaminergic system may contribute to the pathogenesis of neuropsychiatric disorders such as narcolepsy, schizophrenia, depression, AD and PD (Shan et al. 2017). The increased number of histamine neurons in the narcoleptic brain is hypothesized to contribute to the hypnagogic/hypnopompic hallucinations (types of sleep hallucinations that can feel real and often frightening. They can be mistaken for nightmares and can occur during falling asleep, hypnagogic, or awakening, hypnopompic) of this disorder.

A reduction of H_1 receptor binding in the cerebral cortex was observed in AD, depression and schizophrenia, which may imply that H_1 receptor availability is associated with cognitive functions and mood states (Kano et al. 2004, Higuchi et al. 2000). The H_1 receptor knockout animal seems to provide a great opportunity for further studies such an involvement in cognition and anxiety. H_1 receptor antagonists are a potential effective treatment for insomnia (Roth et al. 2007). Although no H_3 receptor inverse agonists has been approved for the treatment of AD, several H_3 receptor inverse agonists have shown therapeutic potential for the treatment of cognitive dysfunction in preclinical studies (Zlomuzica et al. 2016). Preliminary results have shown that H_2 receptor antagonists induced a significant improvement in both positive and negative symptoms of schizophrenic symptoms (Meskanen et al. 2013).

An H_3 receptor inverse agonist, Pitolisant has been approved in 2016 for the treatment of narcolepsy (Syed 2016) and several H_3 receptor antagonists/inverse agonists have already entered Phase II–III clinical trials.

The results describe in this thesis demonstrate that H_3 receptor ligands are good pharmacological tools for the more in-depth study of the histaminergic system in various pathologies including those affecting memory and learning.

Ligands of histamine receptors are among the most used drugs worldwide; hence, understanding the impact of these compounds on learning and memory, mood and anxiety may help improve their pharmacological profile and unravel unexplored therapeutic applications.

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