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Biobehavioural mechanisms of feeding behaviour: associative learning, hedonic preference and motivation

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Tese de Doutoramento em Ciências da Saúde

Trabalho realizado sob a orientação do
Professor Doutor Osborne Almeida
e do
Professor Doutor Nuno Sousa

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إهداءٌ خاص

إلى اَلرَجُل اَلذي رَغَبَ اَلعِلم واَلنّجاح

وإلى المَرأة اَلتي ألهَمَةُ اَلمَسار

The truly great man is he who would master no one,
and who would be mastered by none.
– *Khalil Gibran*

"The simulacrum is never that which conceals the truth
—it is the truth which conceals that there is none.
The simulacrum is true."
Ecclesiastes or *Jean Baudrillard*

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For their trust and unconditional love, for opening for me the way to create my own reality and to search freely for my happiness, for their presence and excitement in every accomplishment and joy. And finally, for showing me that happiness and love are the key of a successful life.

München, May of 2014

Mazen

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Biobehavioural mechanisms of feeding behaviour: associative learning, hedonic preference and motivation

Abstract

The neurobiolgical mechanisms that contribute to the regulation of food ingestion are still incompletely understood, not only because of the complex neural circuitry involved but also because feeding behaviour is regulated through an intricate integration of peripheral and central pathways and signals. Meanwhile, there is much interest in the biological mechanisms underlying overeating and obesity in light of the hypotheses that suggest that obesity and drug addiction are similar diseases, and that obesity is a result of a reward deficency and altered cognitive processing.

This study focused on three major questions: 1) can overweight and obesity be considered a consequence of food addiction? 2) does obesity cause cognitive impairment and reward deficiency? and 3) does the risk of becoming obese change with ageing?

The results of this work add objective evidence for why overeating should not be viewed as an addictive behaviour. Specifically, since sign tracking (ST) in pavlovian conditioning appears to mark vulnerability to compulsive behaviour, and hyper-responsiveness to stress, features that are also seen in drug addiction, we tested whether these characteristics can be generalized to eating behaviour.We found that ST mice are neither prone to overeating or development of obesity and, do not display signs of impaired emotionality or motivation. Second, we showed that the assumption that overeating and drug addiction are similar disorders are likely to be false since obese mice do not display behavioural sensitization to morphine, and conversely, because obese mice that are sensitized to morphine do not over-consume a highly rewarding food.

By testing the influence of overweight and obesity on various key components of feeding behaviour, we showed that increased body mass (adiposity) contributes to diminished interest in learning appetitive tasks but that overweight and obese mice do not suffer from reward deficit syndrome. Accordingly, it is concluded that energy stores, rather than cognitive factors, primarily determine appetitive behaviour.

Lastly, our experiments demonstrated that mice of all ages can form action-outcome as well as stimulus-reward associations, in appetitive associative learning; when given highly-palatable foods

alongside their maintenance diets, aged mice display an ability to adjust their ingestion of both hedonic and standard chows so as to match their usual daily caloric intake.

Our findings are important insofar that they indicate that pharmacological treatments designed for drug abuse are unlikely to be effective at reducing overeating. We show that energetic state dominates over sensory stimuli in the regulation of feeding behaviour in mice, raising awareness about the limitations of extrapolating findings in rodent models to humans. Finally, the results reported in this work suggest that there is lifelong risk for overeating in response to reward cues. However, aged mice appear to be physiologically and behaviourally equipped to avoid becoming obese, in contrast to humans who tend to overeat because of an overriding of physiological signals by hedonic principles in the form of palatable foods and environmental food cues.

Mecanismos biocomportamentais de alimentação: comportamento associativo, preferência hedónica e motivação

Resumo

Os mecanismos neurobiológicos que contribuem para a regulação da ingestão de alimentos ainda não são totalmente conhecidos, não só devido aos circuitos neuronais complexos que estão envolvidos nestes processos, mas também porque o comportamento alimentar é regulado através de uma complexa integração de sinais e sistemas centrais e periféricos. Existe também bastante interesse em compreender os mecanismos biológicos envolvidos em obesidade e excesso de alimentação, uma vez que, cada vez mais, surgem hipóteses que a obesidade e vício em drogas são patologias semelhantes e que a obesidade é o resultado de uma deficiência nos mecanismos de recompensa e de uma alteração no processamento cognitivo.

Este trabalho foca-se em três grandes questões: 1) podem o excesso de peso e obesidade ser considerados uma consequência do vício em alimentos?; 2) a obesidade causa problemas cognitivos e nos mecanismos de recompensa?; e 3) o risco de nos tornarmos obesos altera-se com a idade?

Os resultados deste trabalho indicam objectivamente porque uma alimentação excessiva não deve ser considerada um comportamento aditivo. Especificamente, o seguimento de sinais (do inglês, ST) no condicionamento pavloviano parece ser um indicador de susceptibilidade para comportamento compulsivo e para híper-responsividade para o stresse, características que são também observadas em situações de vício em drogas; testámos se estas características podem ser generalizadas para comportamento alimentar. Observámos que ratinhos ST não apresentam maior propensão para se alimentarem em excesso ou para desenvolverem obesidade, e não apresentam sinais de alteração emocional ou na sua motivação. Mostramos também que a hipótese de vício em drogas e excess de alimentação serem patologias semelhantes é falsa uma vez que ratinhos obesos não apresentam sensibilização comportamental a morfina e, além disso, ratinhos obesos que apresentam sensibilidade a morfina não consomem em excesso alimentos dados como recompensa.

Ao testar a influência do excesso de peso em vários componentes do comportamento alimentar, mostramos que o aumento de massa corporal (adiposa) contribuí para a diminuição do interesse em aprender tarefas relacionadas com o apetite mas que ratinhos obesos não apresentam deficiência no

sistema de recompensa. Em concordância, concluímos que a reserva energética, mais do que factores cognitivos, é o factor mais importante no apetite.

Finalmente, as nossas experiências demonstram que ratinhos de todas as idades têm a capacidade de fazer associações acção – resultado e de estímulo-recompensa, em comportamento associativo de apetite; quando são sujeitos a alimentos com sabor agradável juntamente com a sua dieta normal, ratinhos de maior idade apresentam a capacidade de ajustar a ingestão da dieta normal e melhorada para manter o consumo calórico diário em valores normais.

Os nossos resultados são bastante relevantes, uma vez que indicam que tratamentos farmacológicos destinados ao tratamento de drogas de abuso têm pouca possibilidade de ser um tratamento para o excesso de consumo de alimentos. Mostramos que o estado energético é dominante sobre os estímulos sensoriais na regulação da alimentação de ratinhos, alertando para as limitações de extrapolar as conclusões tiradas com um modelo de roedor para humanos. Finalmente, os resultados deste trabalho sugerem que existem um risco para toda a vida causado pelo excesso de alimentação em reposta a estímulos de recompensa. No entanto, ratinhos de maior idade parecem estar equipados em termos fisiológicos e de comportamento para evitar tornarem-se obesos, contrastando com os humanos, que apresentam tendência a comer em excesso devido a uma sobreposição de princípios hedónicos apresentados na forme de alimentos saborosos em relação aos sinais fisiológicos.

List of Abbreviations

5-HT	Serotonin
α-MSH	α-Melanocyte-Stimulating Hormone
ACC	Anterior Cingulated Cortex
AgRP	Agouti-Related Protein
AP	Area Postrema
ARC	Arcuate Nucleus
BED	Binge Eating Disorders
CART	Cocaine/Amphetamine-Regulated Transcript
CB	Cannabinoid
CCK	Cholecystokinin
CMS	Chronic Mild Stress
CNS	Central Nervous System
CR	Conditioned Response
CS	Conditioned Stimulus
DA	Dopamine
DR	Dorsal Raphe
DS	Dorsal Striatum
DSM-V	Diagnostic and Statistical Manual
EC	Endocannabinoid
FST	Forced-Swim Test
GHS-R	Growth Hormone Secretagogue Receptor
GLP-1	Glucagon-Like Peptide-1
GT	Goal Tracking
HFD	High- Fat Diet
HPA	Hypothalamo-Pituitary-Adrenal
IT	Intermediate Trackers
Icv	Intracerebroventricular
LC	Locus Coeruleus
LepR	Leptin Receptor

LFD	Low-Fat Diet
LH	Lateral Hypothalamus
MCR	Melanocortinn Receptor
MCH	Melanin-Concentrating Hormone
N.acc	Nucleus Accumbens
NC	Normal Chow
NE	Norepinephrine
NO	Novel Object
NPY	Neuropeptide Y
NT	Neurotransmitter
NTS	Nucleus of the Solitary Tract
OF	Open Field
OFC	Orbitofrontal Cortex
PAG	Periaqueductal Gray
PBN	Parabrachial Nucleus
PFC	Prefrontal Cortex
POMC	Proopiomelanocortin
PTN	Pedunculopontine Tegmental Nucleus
PVN	Paraventricular Nucleus
PYY3-36	Peptide YY3-36
SN	Substantia Nigra
SPT	Sucrose Preference Test
ST	Sign-Tracking
VMH	Ventromedial Hypothalamus
VP	Ventral Pallidum
VTA	Ventral Tegmental Area

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CHAPTER 1

General Introduction

1.1 Feeding behaviour and survival

Hunger, fear and procreation are the main drives to balance, reestablish and maintain survival in all species. These drives, manifested as an internal arousal which causes the organism to satisfy its physiological needs, induce basic motivated behaviours to satisfy the needs by fulfillment (eating, copulation) or defense (protection, fight or flight response) to facilitate adaption to the prevailing environment. The organism requires continuous energy supplies for optimal mental and physical performance, giving hunger an important place in the maintenance of life.

Eating different types of food ensures adequate procurement of essential nutrients namely carbohydrates, and fats, as well as vitamins and minerals. Glucose is the key source of energy in mammals. In contrast to peripheral tissues which can derive energy from fat (Wang et al., 2006), glucose is the only form of energy that is directly utilizable by the brain. Fat reserves play an essential role in natural selection and survival; they guarantee energy in difficult times such as winter when food is scarce and also during starvation.

Feeding behaviour is a complex behaviour that depends on different processes such as hedonic evaluation of nutrients, motivation to direct actions toward seeking and consuming food and lastly, the ability to identify energy sources by forming associations between food-related environmental stimuli and nutritional value. These processes rely on the functioning of different brain regions and the inputs from peripheral organs.

1.2 The selfish brain

The concept of the "selfish brain" has been propagated by Achim Peters of the University of Lubbeck-Kiel in Germany (Peters, 2011a), based on the fact the brain is the most "energy-hungry" organ. Although it represents only 2% of total body mass, it uses approximately 20% of the organism's energy supply (Erbsloh et al., 1958). It achieves its demands by regulating feeding behaviour and energy metabolism (Lam et al., 2009). There are various metabolic sensors in the brain, such as insulin receptors (Brüning et al., 2000) and glucose transporters (Chari et al., 2011) that can sense and control peripheral glucose and energy homeostasis. Energy homeostasis is maintained through a

balance between energy intake, storage and expenditure so as to constantly provide fuel to the organs. Feelings of hunger, alertness and explorative behaviour are enhanced and energy expenditure is reduced as blood glucose levels fall below a certain threshold and, as glucose levels rise after a meal, the individual experiences feelings of satiety. These feeding-related behaviours, including the feelings of hunger and satiety are under the control of the brain which itself needs, but cannot directly acquire, energy. Here, from the perspective of the "selfish brain theory" it is interesting to note that in people that die of starvation, peripheral organs such as the heart, spleen, kidney, and liver lose about 40% of their mass, as compared to the brain where the loss is 1% at a maximum (Krieger, 1921); similar observations have been reported in adult and fetal animals (Goodman et al., 1984; Miller et al., 2002; Kind et al., 2005; Peters et al., 2011).

1.3 Brain-body communication in feeding behaviour and homeostatic regulation

Maintaining and regulating energy balance requires communication between different peripheral and brain systems. The vagus nerve provides peripheral neural inputs to the brain, but peripherally-produced hormones that signal energy levels and stores (e.g. leptin), together with nutrients and their metabolites, also play an important role in determining states that will increase feeding (orexigenic) or stop eating (anorexigenic). These signals, together with sensory information (olfactory, gustatory, visual and textural), are integrated in the brain to execute feeding behaviour (or its termination) (Fig. 1.1).

Brain circuitries involved in feeding behaviour

The brain networks involved in feeding behaviour range from those concerned with sensing the organism's instantaneous energetic demands, the sensory and energetic properties of foods (hedonic evaluation), to those that regulate arousal, attention, learning, memory, motivation and motor responses. The neural circuitry involved in feeding and energy regulation was demonstrated using lesioning, morphological and neuroimaging, molecular genetics, and pharmacological approaches in humans and laboratory animals. This brain network requires interaction between different areas and

systems, mainly the visual, olfactory and the somatosensory systems, brainstem nuclei, and the hypothalamus, amygdala, hippocampus, dorsal striatum (DS), nucleus accumbens (N.acc), ventral pallidum (VP), insula, anterior cingulated cortex (ACC), orbitofrontal cortex (OFC), portions of the thalamus and the prefrontal cortex (PFC) (Berthoud, 2011) (Fig. 1.1).

The *brain stem* is implicated in the oral sensory (taste nerves) and the motor events related to the act of eating. It is the primary neuronal and hormonal connection between the gut and the brain. Other than receiving information from the tongue, brain stem nuclei such as the area postrema and the nucleus of the solitary tract (AP/NTS) are innervated by the vagus nerve which carries information about gastric distension (mechanical stretch of the stomach and duodenum); the latter is relayed to the lateral parabrachial nucleus (PBN) (Hellström et al., 2004). In addition the pedunculopontine tegmental nucleus (PTN, cholinergic and glutamatergic projections) (Benarroch, 2013) and other nuclei in the reticular formation produce monoaminergic neurotransmitters that are involved in modulating functions related to feeding behviour, including arousal, pleasure, motivation, mood regulation, motor execution and energetic homeostasis: ventral tegmental area (VTA, dopaminergic projections), the locus coeruleus (LC, noradrenergic projections), dorsal raphe nucleus (DR, serotoninergic projections) (Dahlström and Fuxe, 1964).

The *hypothalamus* is massively connected with the periphery and emotional and cognitive brain regions, playing a central role in regulating primitive functions that are essential for survival. Among others, it regulates glucose and energy homeostasis and eating behaviour. Three hypothalamic nuclei are prominently involved in these functions: the paraventricular nucleus (PVN), the lateral hypothalamus (LH) and the arcuate nucleus (ARC) in the ventromedial hypothalamus (VMH) (Lam et al., 2009). The ARC contains groups of peptidergic neurons that exert either anorexigenic or orexigenic actions. The anorexigenic group consists of neurons that co-express pro-opiomelanocortin (POMC) and cocaine/amphetamine-regulated transcript (CART). The POMC/CART neurons have an inhibitory action on LH orexigenic neurons and a stimulatory action on anorexigenic neurons in the PVN and AP/NTS. The orexigenic neurons in the ARC co-express the peptides neuropeptide Y (NPY) and agouti-related

protein (AgRP) which exert stimulatory actions on orexigenic neurons in the LH, and inhibitory actions on anorexigenic neurons in the PVN (Hahn et al., 1998; Elias et al., 1999).

The emotional and cognitive brain regions listed above are modulated either directly or indirectly by peripheral signals (Fig. 1.1). The interplay between these regions and the periphery form the core circuitries that are responsible for the regulation of eating, reward expectancy, appetite, learning about energetic contents of foods, contextual memory, hedonic reactions to food, motivation and executive behaviours, hedonic evaluation of stimuli, and associative learning. (Mogenson et al., 1980; Scott and Plata-Salamán, 1999; Gottfried et al., 2003; Kringelbach et al., 2003; Kelley et al., 2005; Rolls, 2005; Berthoud, 2011; Dagher, 2012).

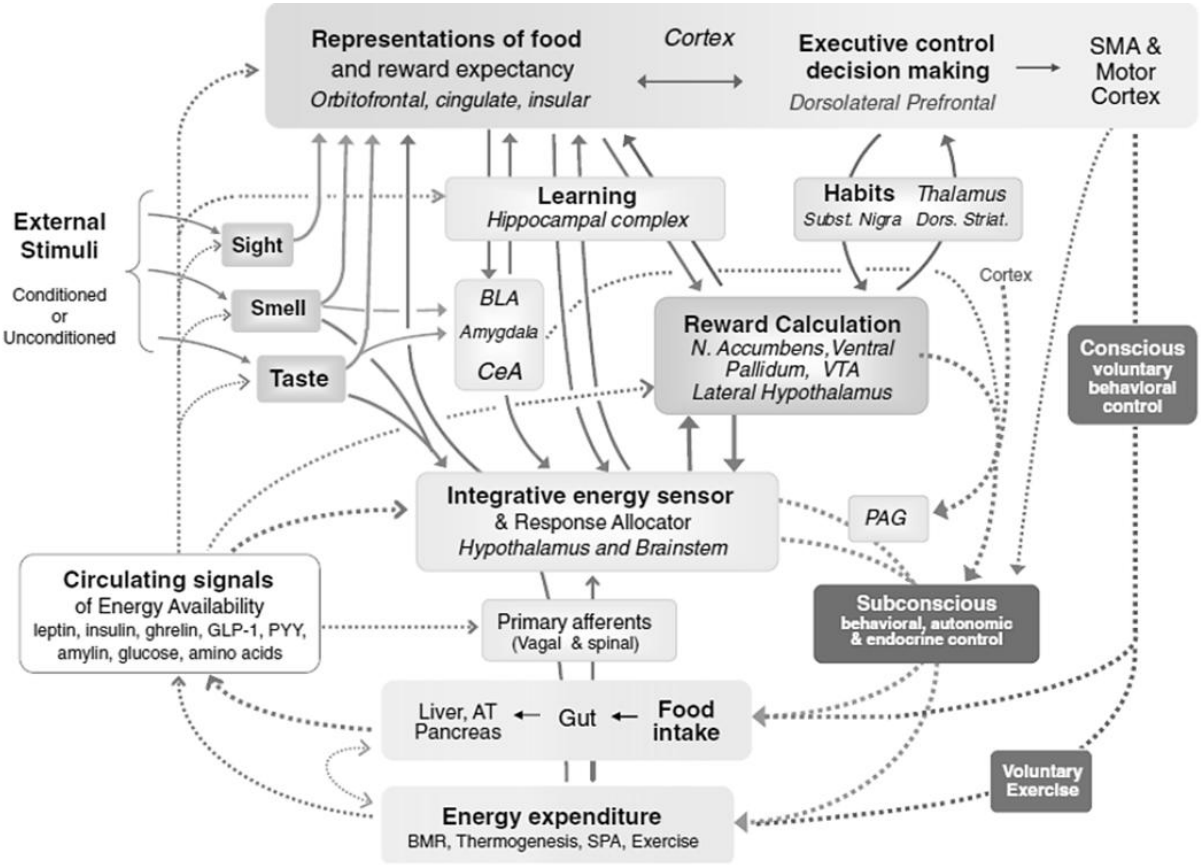

Figure 1.1 Schematic representation of the brain-body communication in the regulation of feeding behaviour and energy homeostasis. The interactions between classical homoeostatic energy regulatory system in the hypothalamus and brainstem and cognitive/emotional brain systems are depicted. Modulation of these regions is accomplished by circulating peripheral hormones and external stimuli. These signals, on one hand, inform about the availability of energy and on the other, influence memory formation, arousal, hedonic evaluation, decision making and voluntary behavioural control. N. Accumbens, nucleus accumbens; SMA, supplemental motor area; BLA, basolateral amygdala; CeA, central nucleus of the amygdala; VTA, ventral tegmental area; PAG, periaqueductal gray; GLP-1, glucgon-like-peptide-1; PYY, peptide YY; AT, adipose tissue; SPA, spontaneous physical activity. (*from: Berthoud, 2012*).

Peripheral regulatory signals

The hormonal signals from the periphery inform the brain about the energy state (actual and in reserve) of the organism. These signals are carried to the central nervous system (CNS) via the bloodstream where they bind to and activate specific receptors in discrete brain regions. Some of the major peripheral chemical messengers related to energy balance and feeding are described briefly below:

Ghrelin is a gut-derived peptide (MW 3370.9), secreted by endocrine cells in the stomach and proximal small intestine. It is the only peripheral orexigenic hormone with central actions (Tschop et al., 2000; Druce et al., 2006). Ghrelin induces feelings of hunger, initiates eating and decreases energy expenditure (Cummings et al., 2001, 2004), but can also increase blood glucose levels by inhibiting insulin secretion (Reiner et al., 2003). Ghrelin binds to the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999) which is expressed by many hypothalamic nuclei but mainly by NPY/AgRP orexigenic neurons in the ARC, PVN, ventromedial nucleus and LH (Guan et al., 1997). Besides increasing appetite, ghrelin plays an important role in various aspects of feeding such as motivation, hedonic feeding, and memory. A comparative study in rats and mouse revealed the GHS-Rs expression in the hippocampus, and many nuclei of the brainstem such as the NTS, DR, PTN, VTA and the substantia nigra (Zigman et al., 2006). Results of studies using peripheral and central injections of ghrelin and ghrelin antagonists in animals have suggested a role for ghrelin in the enhancement of certain forms of memory (Diano et al., 2006), increased motivational drive to work and consume foods whose palatability is increased by their high sugar and/or fat content (Egecioglu et al., 2010; Perello et al., 2010; Skibicka et al., 2012), even if their caloric content is low (Disse et al., 2010). Intra-VTA ghrelin injections in rats increase food intake (Naleid et al., 2005), especially that of palatable food (Egecioglu et al., 2010) and, intravenous ghrelin in healthy humans, evokes responses in brain areas involved in motivation, memory, and hedonic evaluation and appetitive behaviour (amygdala, insula, striatum and orbitofrontal cortex) when pictures of food are presented (Malik et al., 2008).

Leptin is a polypeptide hormone (MW 16240), secreted by adipose tissue. It is a major anorexigenic hormone that controls feeding behaviour and energy homeostasis, mainly by reducing

food intake and increasing metabolism (Campfield et al., 1995; Halaas et al., 1995). During food restriction and starvation, leptin secretion and energy expenditure are diminished (Ahima et al., 1996; Zhang et al., 1994), and in the satiated state, serum leptin levels correlate positively with amount of adipose tissue (Hassnik et al., 1996). Leptin receptors (LepR) are expressed centrally and peripherally; those in the CNS are responsible for mediating the anorexigenic effect of leptin. More specifically, the LepRb expressed in the PVN and on POMC/CART anorexigenic (stimulated by leptin) and NPY/ AgRP orexigenic neurons (inhibited by leptin) in the ARC contribute importantly to the regulation of energy homeostasis (Cheung et al., 1997; Wilson et al., 1999). Leptin receptors in brain regions such as the hippocampus, LH, NTS, and the VTA influence hedonic and motivational aspects of feeding behaviour (Fulton et al., 2006; Funahashi et al., 2003). Intra-VTA, intra-NTS (Kanoski et al., 2014) and intracerebroventricular (icv) leptin injections decrease motivation to work to obtain and consume palatable foods (Figlewicz et al., 2006; Morton et al., 2009). Supporting a role for leptin in regulating the hedonic response to food, a neuroimaging study revealed that humans with a rare genetic deficiency of leptin show an exaggerated striatal response to food images compared to healthy subjects (Farooqi et al., 2007). Lastly, leptin receptors are implicated in memory formation and consolidation in appetitive learning, e.g. conditioned place preference test in rats (Figlewicz et al., 2001) and fear learning in the footshock avoidance test in mice (Farr et al., 2006).

Insulin is a polypeptide hormone (MW 5808), secreted by pancreatic β-cells. It controls glucose homeostasis by promoting glycogenesis and tissue (e.g. muscles, liver, adipose tissue) uptake of glucose. Like leptin, insulin exerts anorexigenic effects by controlling food intake and metabolism (Mcgowan et al., 1993.). The actions of insulin are mediated centrally by insulin receptors on hypothalamic (ARC) POMC/CART and the NPY/AgRP neurons (Marks et al., 1990). When administered centrally, insulin decreases food intake and body weight, mainly through it is action on the hypothalamus, (Woods et al., 1979; McGowan et al., 1992; Brown et al., 2006) and VTA (Mebel et al., 2012) which also expresses insulin receptors (Figlewicz et al., 2003).

Peptide YY3-36 (PYY3-36) is an anorexigenic hormone (MW 4049.5) secreted from the gastrointestinal tract. It levels increase slowly during eating and remain high for several hours after;

during fasting PYY3-36 levels are low (Adrian et al., 1985). The anorexigenic actions of the peptide are mainly mediated through actions on orexigenic NPY neurons in the ARC after its binding to Y2 autoreceptors and inhibition of NPY secretion (Batterham et al., 2002).

Glucagon-like peptide-1 (GLP-1) (MW 4111.5) is also secreted by the gastrointestinal tract; it is released at the beginning of a meal and its levels remain high for an extended period after ingestion. GLP-1 exerts anorexigenic effects by modulating glucose homeostasis (inhibits secretion of glucagon, which its effect is opposite to that of insulin), inhibiting gastric emptying, increasing leptin signaling in the ARC (Gotoh et al., 2005), and exciting vagal afferent neurons to activate an ascending pathway leading to inhibition of food intake (Dockray, 2014). Food intake is reduced after either central or peripheral GLP-1 administration (Tang-Christensen et al., 1998).

Cholecystokinin (*CCK,* MW 1063.2) is an anorexigenic hormone that plays a role in reducing food intake, by inhibiting gastric emptying, stimulating the vagus nerve and activating neurons in the NTS. It is secreted from the duodenum in response to a caloric load from ingested nutrients (Dockray, 2009). Peripheral administration of CCK induces reduction in food intake, meal size (Gibbs et al., 1973; Moran et al., 1993) and operant motivated behaviour to collect food reward (Babcock et al., 1985).

Amylin (MW 3906.3) is an anorexigenic peptide, co-released with insulin from pancreatic β-cells when nutrients are detected in the digestive tract (Kahn et al., 1990). Acute (Chapman et al., 2007; Lutz et al., 1994) and chronic (Bello et al., 2008) peripheral administration of amylin results in reduced food intake. Amylin exerts its anorexigenic effect (Rushing et al., 2001) by binding to its receptors on a variety of regions in the CNS (Beaumont et al., 1993). It has been proposed that its regulatory effects on feeding are mediated by the AP/NTS (Lutz et al., 1998). Amylin was recently shown to act in the VTA to modulate the motivation to work for food in an operant task by reducing the intake of standard food and palatable sucrose solution (Mietlicki-Baase et al., 2013).

Central regulatory signals and mediators

Orexin (or hypocretin) A (MW 3561.1) *and B* (MW 2936.4) are peptides produced by neurons of the LH, partially overlapping with MCH neurons (see below). By definition, orexin neurons are orexigenic and constitute an important component in the hypothalamic circuit controlling food intake and energy balance (Sakurai et al., 1998). Interestingly, icv injection of orexin A stimulates food consumption under both fasted and satiated states (Shiraishi et al., 2000) whereas antagonism of orexin receptors decreases food intake (Haynes et al., 2000). Their primary innervation comes from the ARC. The orexin system is also involved in arousal, wakefulness, response to stress, energy expenditure, learning and memory and rewarding/motivational aspect of feeding (Mieda and Yanigasawa, 2002). Orexin neurons send excitatory signals to a range of brain areas, innervating dopaminergic (VTA), noradrenergic (LC), serotoninergic (DR), histaminergic (tuberomammillary nucleus), acetylcholinergic (PTN) cell bodies, hypothalamic nuclei (ARC, dorsomedial hypothalamus, PVN) as well as the N.acc, ventral pallidum (VP), amygdala and parts of the PFC (Marcus et al., 2001) (fig 1.3). The LH is involved in detecting food stimuli and initiating behaviours related to feeding. Stimulation of appetite and preparation for food arrival by orexin reflects integration of sensory (sight, smell, taste of food), chemical (gastric acid secretion) and physical (gut motility) signals in the LH from where orexin neurons project to the dorsal motor nucleus of the vagus (Zbigniew et al., 2001).

In addition, orexin neurons are sensors of central circulating glucose, being activated when glucose levels are low or inhibited after vagal stimulation of the NTS when food is present in the gastrointestinal tract (Williams et al., 2004). Orexin neurons are also inhibited and stimulated by leptin and ghrelin, respectively; receptors for both of the latter peptides are present in the LH (Beck et al., 1999; Komaki et al., 2001).

Orexin has also been reported to influence motivational and hedonic aspects of feeding, being implicated in food seeking, conditioned behaviours and in motivation to work for standard and highly-palatable foods (Choi et al., 2010; Cason and Aston-Jones, 2013). Recent studies using central microinjections of orexin demonstrated the presence of "orexin hotspots" in the posterior part of the VP, a region implicated in hedonic "liking" reactions to food (e.g. sucrose) (Ho and Berridge, 2013).

Melanin-concentrating hormone (MCH). This peptide (MW 2386.9) is expressed in LH neurons that are innervated by ARC neurons that release α-MSH, CART, NPY, AgRP; the MCH neurons themselves project to the VMH, NTS, N.acc, some parts of the PFC, and the LC (Griffond and Risold, 2009) (Fig. 1.2). The MCH system is involved in consumption and motivation to feed; thus, icv administration of MCH enhances appetite in sated rats, and increases acute and chronic food intake (Della-Zuana et al., 2002). This role of MCH is supported by the observation that an antagonist of MCH in obese mice reduces food intake and body weight (Mashiko et al., 2005).

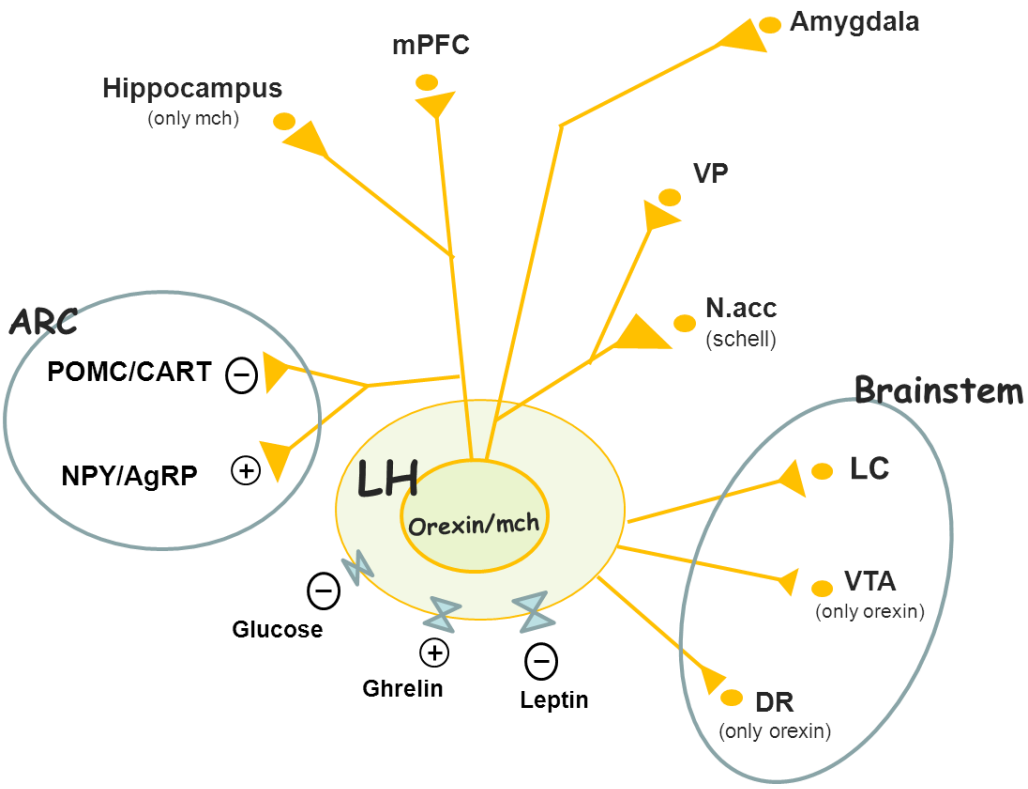

Figure 1.2 Schematic drawing of the mechanisms by which neurons containing orexin and MCH in the lateral hypothalamus (LH) regulate feeding behaviour. These neurons are regulated by metabolic sensors: leptin and ghrelin receptors, and glucose transporters. When energetic demand increases, the LH stimulates NPY/AgRP neurons and inhibits POMC/CART neurons in the arcuate nucleus (ARC) to increase food intake; at the same time, orexin/MCH neurons project to dopaminergic (VTA), noradrenergic (LC) and serotoninergic (DR) nuclei in the brainstem and to cognitive/emotional brain regions including the nucleus accumbens (N.acc), ventral pallidum (VP), amygdala, hippocampus, and the medial prefrontal cortex (mPFC) where they increase arousal, attention, exploratory behaviour, food seeking behaviour, incentive salience and hedonic evaluation of stimuli.

Melanocortin receptors (MCR). The melanocortin peptides are cleaved from pro-opiomelanocortin (POMC) neurons in the ARC. One of these, α-melanocyte-stimulating hormone (α-MSH) (MW 1664.8) is implicated in feeding and energy homeostasis through its action via melanocortin-3 (MC3R) and -4 (MC4R) receptors (Smith and Funder, 1988; Lerner, 1993). Intracerebroventricular administration of α-MSH decreases food intake and stimulates energy expenditure (Fan et al., 1997) and activation of MC4R in the PVN and LH induce satiety. Consistent with these observations, MC4R deficient mice eat more and display disrupted metabolism and excessive body weight gain (Huszar et al., 1997; Marsh et al., 1999). Very recently, it was reported that the MCR may also act in the mesolimbic dopamine system: intra-VTA injection of an MCR antagonist in rats was seen to enhance food intake and body weight over 24 hours, with opposite effects being observed with an MCR agonist (Roseberry et al., 2013)

Endocannabinoids (ECs). The endocannabinoid (endogenous cannabinoid, EC) system is involved in many brain and peripheral functions and pathologies. It is implicated in the regulation of emotions, stress, anxiety, reward, energy homeostasis and food intake. The endogenous ligands (anandamide and 2-arachidonoylglycerol) bind to cannabinoid (CB of which there are two subtypes, CB1 and CB2) receptors expressed in peripheral (gut, stomach, liver, gut, adipose tissue, pancreas, skeletal muscle) and central tissues where they modulate hormone and neurotransmitter actions and metabolic processes. For example, EC contribute to the control of lipid and glucose metabolism, modulate the release of insulin and glucagon, and regulate the synthesis and secretion of various peripheral anorexigenic and orexigenic hormones (Bermudez-Silva et al., 2010). Central CB1 receptors, expressed in the NTS, PVN, ARC, and LH, are implicated in food intake and energy expenditure; those expressed in the VTA, LC, amygdala, and the N.acc are implicated in motivational aspects of feeding (Flores et al., 2013). In general, CB1 agonists are orexigenic (Soria-Gomez et al., 2007). Central EC release is low after feeding and high during hunger, and are subject to modulation by stress hormones (glucocorticoids) and peripheral metabolic signals (Malcher-Lopes et al., 2006; Kola et al., 2008).

Oxytocin and vasopressin. These two peptides are secreted mainly from the PVN, and released peripherally and centrally. They are involved in a variety of functions, like maternal physiology (fetal

expulsion, lactation), water homeostasis and also regulating food intake and energy balance (for review: Sabatier et al., 2013). They exhibit an anorexigenic effect through their central targets in the ARC and the VTA. It has been shown that the vasopressin (MW 1084.3) peripheral administration (Meyer et al., 1989) and the oxytocin (MW 1007) peripheral and central (icv) administration induce suppression of food intake in short-term (Arletti et al., 1989), but decrease in a long-term body weight and feeding in a dose-dependent manner in normal and obese animals (Morton et al., 2012). Also, the intra-VTA oxytocin administration result in a decrease of palatable food (10% sucrose solution) consumption (Mullis et al., 2013).

While peptides make an important contribution to the regulation of feeding behaviour and metabolism, feeding behaviour depends crucially on primitive neurotransmitters (norepinephrine, dopamine and serotonin), as described below.

Norepinephrine (NE) is a monoaminergic neurotransmitter that plays a role in a variety of emotional and cognitive processes, including alertness, arousal, anxiety, fear and stress response, learning, memory formation, and appetitive behaviours. It was the first neurotransmitter to be studied in the context of regulation of food intake (Grossman, 1960). Intracerebroventricular and intra-hypothalamic application of NE causes a strong rise in feeding (Kruk, 1973), effects mediated by α2- and β-adrenergic receptors in the PVN (Leibowitz, 1988). On the other hand, activation of α1 adrenergic receptors by NEergic agonists inhibit food intake (Davies and Wellman, 1992).

The LC, located in the brain stem, is the main NE-producing nucleus, sending projections to a wide range of cognitive/emotional brain areas (Samuels and Szabadi, 2008) (Fig. 1.3). These cell bodies have glucosensing proprieties (Illes et al., 1994) and express leptin receptors (Hay-Schmidt et al., 2001). The NE system plays a major role in evaluation of the hedonic and reinforcing properties of natural rewards and drugs of abuse, partly because of its anatomical and functional connections to dopaminergic reward pathways (Moore and Bloom, 1979); specifically, noradrenergic LC neurons directly and/or indirectly innervate the VTA and N.acc as well as the PFC (Grenhoff et al., 1993) (Fig. 1.5). In an interesting study (Ventura et al., 2008) showed that the amount of NE released in the medial PFC correlates positively with the incentive salience of palatable foods.

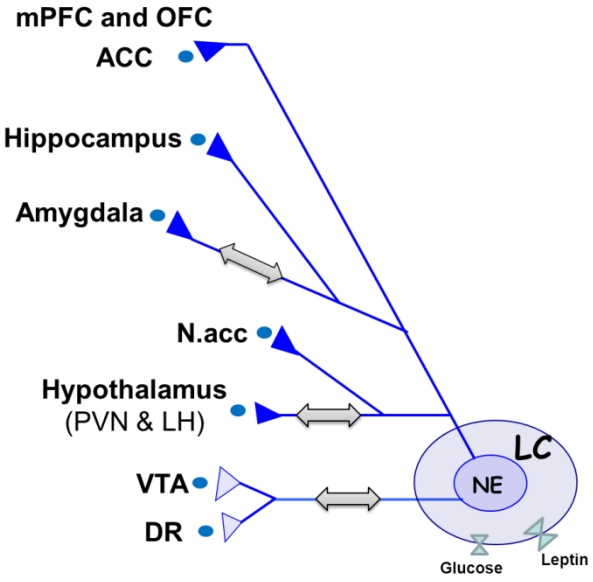

Figure 1.3 Schematic drawing of norepinephrine (NE) projections from the locus coerulus (LC) to wide range of cognitive/emotional brain areas. These neurons contain metabolic sensors: leptin receptors, and glucose transporters, and they are regulated through dopamine (VTA) and serotonin (DR) innervation from the brainstem, as well as the paraventricular nucleus (PVN), lateral hypothalamus (LH) and amygdala. The LC regulates the activity of the VTA, DR and of cognitive/emotional brain regions, including the PVN, nucleus accumbens (N.acc), amygdala, hippocampus, medial (mPFC) and orbitofrontal cortex (OFC), and the anterior cingulated cortex (ACC). All these projections are implicated in alertness, arousal, emotional memory formation and retrieval, autonomic response to stress, behavioural activation and exploration, and regulating feeding behaviour.

Serotonin (5-HT). Serotonin is a monoaminergic neurotransmitter produced in a large group of cell bodies in the brainstem, referred to as the dorsal raphe (DR). The DR projects to various brain regions including the hypothalamus, N.acc, VTA, amygdala, hippocampus and the PFC (Dahlström and Fuxe, 1964; O'Hearn and Molliver, 1984) and to the LC (Fig. 1.4). The 5-HT system is involved in a variety of functions such as sleep regulation, mood, and pain. In addition, it is implicated in some motivational aspects of natural and drug-related rewards, mainly through direct interactions with dopaminergic pathways in the VTA, DS and N.acc and indirect ones involving the PFC (Azmitia and Gannon, 1986; Alex and Pehek, 2007) (Fig. 1.5). Interestingly, DR cell bodies express ghrelin (Zigman et al., 2006) and leptin receptors (Hay-Schmidt et al., 2001).

Numerous studies examined the effect of 5-HT on food intake. Generally, 5-HT is suggested to play a role in body weight and increased satiety, its anorexigenic effects being mediated by 5-HT2c receptors in the ARC; the latter was demonstrated by injecting 5-HT2c agonists and antagonists into the hypothalamus (Liebowitz et al., 1993; Wong et al., 1988).

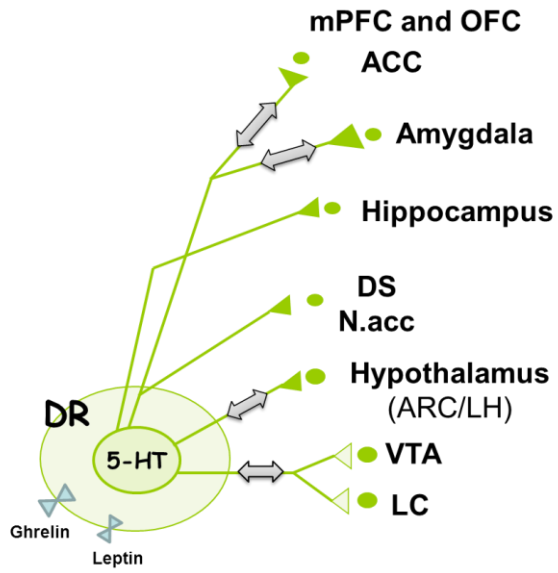

Figure 1.4 Schematic drawing of serotonin (5-HT) projections from the dorsal raphe (DR) to a range of cognitive/emotional brain areas. These neurons carry metabolic sensors, namely, leptin and ghrelin receptors. The 5-HTergic cell bodies are regulated by dopamine (VTA) and norepinephrine (LC) innervation from the brainstem, also by other inputs from the medial prefrontal cortex (mPFC), amygdala and hypothalamic nuclei such as the arcuate nucleus (ARC) and the lateral hypothalamus (LH). In turn, DR regulates the activity of the VTA, LC and of brain regions such as the ARC and LH that are concerned with eating and metabolic homeostasis and cognitive/emotional brain regions such as the dorsal striatum (DS), nucleus accumbens (N.acc), amygdala, hippocampus, mPFC, orbitofrontal cortex (OFC), and the anterior cingulated cortex (ACC). All these projections are implicated in attention, alertness, emotional learning, memory, mood and stress response, appetite and food intake regulation.

Dopamine (DA), like NE is a monoaminergic (catecholaminergic) neurotransmitter. It is mainly produced in two large groups of cells in the brainstem (VTA) and the substantia nigra (SN). The extensive projection of the VTA to limbic and cortical areas, namely the N.acc, amygdala, and PFC is called the mesocorticolimbic pathway; The SN projects mainly to the dorsal striatum along the mesostriatal pathway (Bentovoglio and Morelli, 2005). The dopaminergic system is implicated in many functions, including motor activation, arousal, attention, anticipation, emotion, learning, memory, rewarding and motivational aspects of natural (food, sex, sociability) and synthetic (substances and drugs of abuse) rewards, but also in fear, pain, and aversive reactions (Robbins and Everitt, 1982; Salamone, 1994). DA also plays an important role in feeding-related behaviours. It was first considered that DA release in the N.acc is responsible for the hedonic response (liking) to pleasant stimuli, i.e. that it was "neurotransmitter of pleasure", but the exact role of dopamine in the reward system was only later elucidated.

Pioneering work by Berridge and Robinson showed that liking reactions to sweet taste were not affected by lesions of dopaminergic neurons innervating the N.acc and the DS (Berridge and Robinson, 1998). Other studies confirmed this by showing that neither pharmacological blockade of DA neurotransmission nor the absence of DA (using *DD* mice which cannot synthesize dopamine) influenced the hedonic reactions to sucrose (Kaczmarek and Kiefer, 2000; Cannon and Palmiter,

2003). On the other hand, other investigations revealed the importance of DA in novelty, formation of stimuli-reward associations (Schultz, 1997) and, importantly in (i) motivation (wanting) to work for food (Wyvell and Berridge, 2000) and (ii) attribution of motivational valence (incentive salience) to reward-related cues (Ikemoto and Panksepp, 1999; Berridge and Robinson, 1998).

The main circuits involved in the reinforcement of appetitive behaviours are the VTA projection to the N.acc and the VP; other regions that regulate the activity of the N.acc and implicated in reward, learning, incentive salience are the amygdala, DS, hippocampus, PFC, LC and DR (Berridge and Kringelbach, 2008.). And, as already mentioned previously, the activity of the VTA can also be modulated not only by sensory stimuli (taste, smell, sight) but also by other central signals (NE, 5-HT, orexin) and metabolic signals arising in the periphery (leptin, ghrelin, insulin), which together, act in a coordinated fashion to control feeding behaviour and caloric balance (Grenhoff et al., 1993; Figlewicz et al., 2003; Naleid et al., 2005; Alex and Pehek, 2007; Egecioglu et al., 2010; Kanoski et al., 2014) (Fig. 1.5).

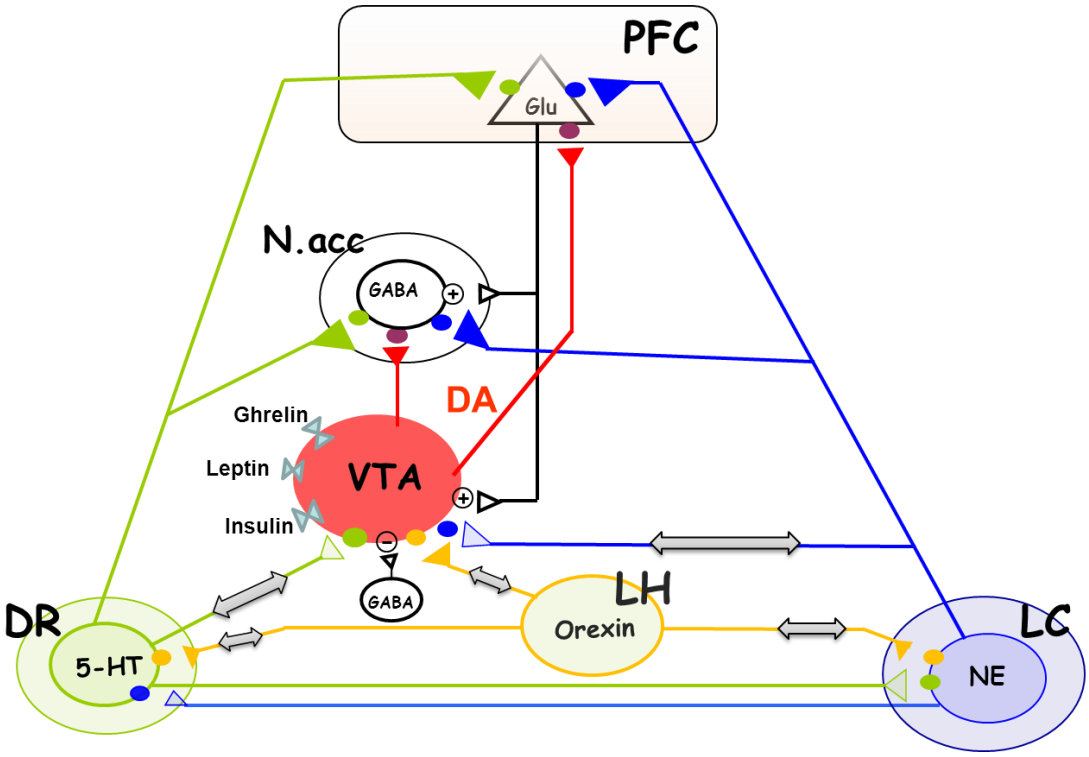

Figure 1.5 Schematic drawing of the pathways and mechanisms by which peripheral hormones and neurotransmitters regulate dopamine (DA) neurons in the ventral tegmental area (VTA), and the nucleus accumbens (N.acc). The VTA is regulated by metabolic sensors (leptin, ghrelin and insulin receptors) and by other neurotransmitters such as the norepinephrine (NE), serotonin (5-HT), orexin, γ-Aminobutyric acid (GABA) and glutamate (Glu). Neurons of the locus coerulus (LC), dorsal raphe (DR), VTA and the lateral hypothalamus (LH) are coupled and regulate each other in a bidirectional manner. In addition, with the prefrontal cortex (PFC), they exert regulatory control over the N.acc.

Opioids. The endogenous opioid system consists of three families of peptides, the endorphins, enkephalins, and dynorphins, whose actions are mediated by μ, δ and κ receptors, respectively. Opioid receptors, widely expressed in the peripheral and central nervous systems, are implicated in a wide range of functions, including the response to stress and pain, emotions, reward, sexual activity, attachment behaviour, and autonomic control (Bodnar, 2010; Nandhu et al., 2010). In particular, endorphins are strongly involved in the rewarding aspects of food intake. β-endorphin, synthesized from pro-opiomelanocortin (POMC) neurons in the ARC and NTS is released at sites in the brainstem, spinal cord and all limbic areas, including the N.acc, VP, amygdala, hippocampus, VTA and periaqueductal gray (PAG) (Le Merrer et al., 2009). Studies in humans revealed that the endogenous opioid system plays an important role in enhancing preference, hedonic taste reactions and the intake of foods that are high in fat and sugar (Drewnowski et al., 1992, 1995). These findings were supported by studies in animals (Marks-kaufmann et al., 1985; Islam and Bodnar, 1990); specifically, endogenous opioids (β-endorphin) acting on μ opioid receptors within the VTA, N.acc and the amygdala enhance food intake and motivation to work for palatable foods (McBride et al., 1990; Zhang et al., 1998, 2003; Kim et al., 2004; Baldo et al., 2005; Mahler and Berridge, 2012). More recently, opioids were shown to regulate feeding, mainly by enhancing hedonic reactions or "liking" of foods, with opioid-responsive hedonic hotspots in the N.acc, and the VP being identified (Peciña and Berridge, 2000, 2005; Smith and Berridge, 2005). Thus, the current view is that the mechanisms underlying hedonic liking which are opioid-dependent, can be dissociated from those underlying incentive salience or motivation (DA- and opioid dependent) (Barbano and Cador, 2007; Berridge, 2009; Kringelbach and Berrdige, 2009; Richard et al., 2013) (Fig. 1.6).

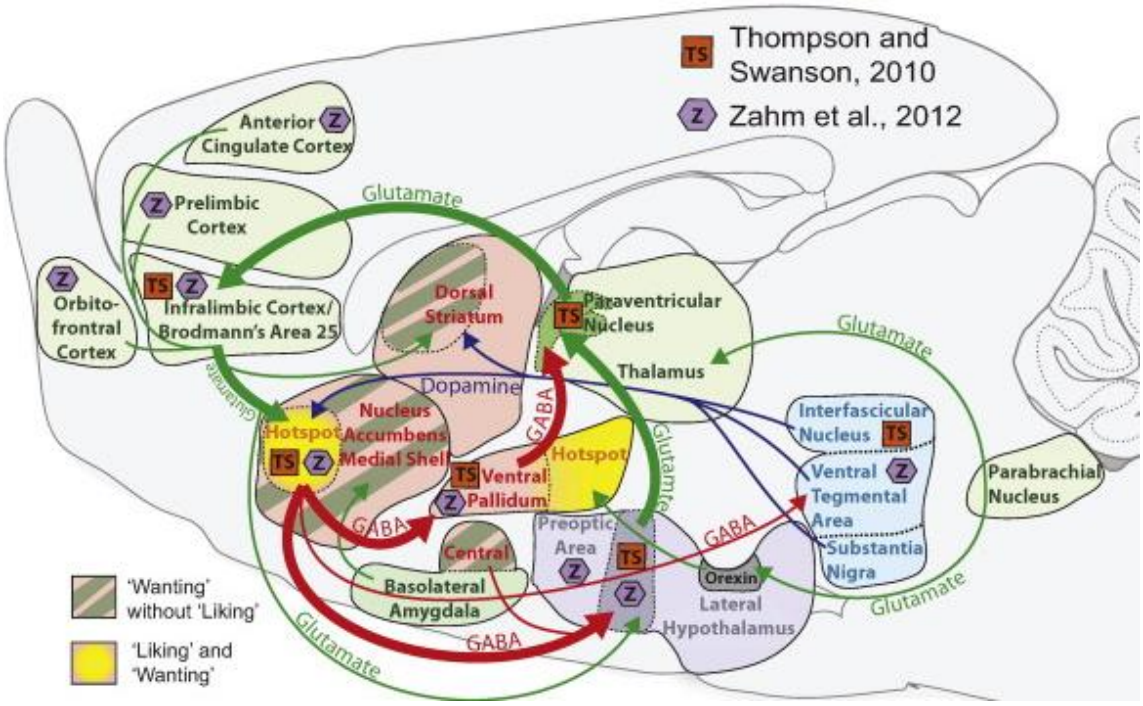

Figure 1.6 Neural circuits underlying motivated 'wanting' and hedonic 'liking'. A summary map showing the connections between cortical, limbic, and midbrain nuclei, with a particular focus on the unique connectivity of the N.acc hotspot. Thompson and Swanson (TS, red boxes; 2010) reported that the N.acc hotspot is embedded in a closed-circuit loop, receiving corticolimbic inputs from infralimbic cortex, and projecting outputs to restricted subregions of hypothalamus (lateral preoptic area–lateral hypothalamic transition zone) and rostral ventral pallidum. These hypothalamo-pallidal afferents then project to paraventricular nucleus of the thalamus, which then completes the loop by sending efferents to infralimbic cortex. Zahm et al. (Z, purple hexagons; 2012) suggest additional connectivity in a pattern similar to lateral septum. GABAergic projections are indicated in red, hedonic hotspots are marked in yellow, potentiated 'wanting' (without 'liking') regions are indicated by dark green stripes, glutamatergic projections are bright green, and dopaminergic projections are marked in blue. (*from: Richard et al., 2013*).

1.4 Excessive feeding: a result of the brain being hijacked by hedonic hunger

Evolutionarily-conserved feeding behaviour has been challenged in industrialized societies which have easy-to-obtain abundant supplies of food and, at the same time, expend less energy and time in physical activity because of lifestyle and technological factors. A serious consequence of these changes appears to be the primary cause for the present worldwide obesity epidemic. At the same time, modern societies are influenced by the food industry which has used scientific knowledge to make foods more attractive in terms of smell, taste and colour, and new technologies to reduce food prices, increase portion-size and extend shelf-life. Thus, cheap, highly-palatable and calorie-rich foods now carry more incentive salience (Wansink and Kim, 2005; Wansink et al., 2006). Added to this, healthy foods are more expensive and healthy eating habits do not enjoy the same media promotion that less-healthy, but convenient, foods receive (Powell et al., 2007; Mink et al., 2010; Corsini et al., 2011). All of above-mentioned industry-exploited tactics are based on knowledge of human preferences and behaviours that depend on behavioural conditioning. Notably, children, who are most susceptible to conditioning, are usually the primary targets of advertising and other promotional actions (Jones et al., 2010; Powell et al., 2010; Hebden et al., 2011; Boyland et al., 2012; Pettigrew et al., 2012).

Hedonic foods

The high levels of fat and sugar in highly palatable and energy-dense foods stimulate food intake, accumulation of body fat and subsequently, body weight gain (Sclafani and Springer, 1976; Ramirez and Friedman, 1990). Preference for such foods is attributable to the rewarding components of fatty and sweet foods (Levine et al., 2003; Scalafani, 2004); it is therefore not surprising that, the easy availability of such foods leads to their overconsumption (Tordoff, 2002; Rolls et al., 2006).

Representation of pleasantness occurs in the insula cortex, whereas taste, smell and sight are represented in the orbitofrontal cortex (OFC) (Scott et al., 1986; Verhagen et al., 2004). These regions are implicated in attributing affective salience to foods, but their activity is modulated by metabolic state, such that the actions that they trigger vary under states of hunger and satiety (Rolls et al., 1986, 1989; Critchley and Rolls, 1996). It is important to note however, that fat and sugar exert their rewarding and motivating effects independently of each other (Freed and Green, 1998; Sclafani and

Ackroff, 2003), and generally, greater preference is shown when the two types of food are combined (Kimura et al., 2003). Besides their sensory properties, caloric content adds to the liking of fat and sugar; the caloric content of foods is sensed by the taste buds as well as gastrointestinal chemosensors (taste and nutrient receptors). These peripheral calorie detectors transmit information to brain regions involved in metabolic homeostasis to enhance (and eventually, to suppress) appetite and motivation (Sclafani and Ackroff, 2012). Interestingly, hungry rats can discriminate between two rewarding foods on the basis of caloric content and show preference for calories over sensory qualities (hedonic value) of the foods (Meriel and Bolles, 1988). Other studies in mice showed a diminution in the consumption and the motivation to obtain foods that are uncoupled from energy content (Beeler et al., 2012); on the other hand, even after genetic ablation of taste receptors, mice were able to show similar preference as control ones for a sweetened solution, and also dopamine release in the N.acc (De Araujo et al., 2008). In summary, while the hedonic content of foods contributes to feeding behaviour, hedonic components alone are not sufficient to stimulate food intake; caloric content of food plays a dominant role in this respect.

Conditioned food intake overrides metabolic homeostasis

Energy and sensory enhancers in food, together with commercial promotion and anecdotal reports by peers, are thought to contribute to the overriding of metabolic controls over feeding by the brain. This means that individuals may consume foods even when the body has sufficient energy and nutrient reserves. Many studies have investigated the influence of food cues on eating behaviour; they have shown for example, that T.V. food commercials can have a major impact on the preference and the consumption of energy dense foods in children (Halford et al., 2004; Boyland et al., 2011) and be causally related to body weight gain in adults of both sexes (Blass et al., 2006; Bowman, 2006; Hu et al., 2001, 2003). Similarly, learned cues (conditioned stimuli, CS+) can lead to an overriding of satiety cues and potentiate conditioned food intake (Weingarten, 1983; Cornell et al., 1989; Petrovich et al., 2007b).

Associative learning is an evolutionarily-conserved adaptive behaviour; in the context of feeding, it enables individuals to form associations between neutral stimuli and stimuli with nutritional (e.g. energetic) value. Like hedonic preference (liking) and wanting (motivation), associative learning is an

indispensable component of food reward (Fig. 1.7). Pavlovian (classical) and operant (instrumental) conditioning are the two main forms of associative learning. Different brain regions are involved in this appetitive conditioning, mainly areas activated by ghrelin (Van der Plasse et al., 2013), but also others implicated in memory formation, motivation, reward, and anticipation. The mere exposure to food stimuli (e.g. smell, sight) can increase feelings of hunger and stimulate appetite (Bossert-Zaudig et al., 1991), salivation (Bayliss, 1916; Klajner et al., 1981; Christensen, 1983) and insulin release (Woods, 1991); all of these represent anticipation of ingestible food. Experiments have shown that triggering conditioned appetitive action after pairing food cues with calories is mediated by a brain network that includes the amygdala, LH, N. acc, medial PFC and dopamine release (Petrovich et al., 2002, 2005, 2007). These and other studies also demonstrated the role of environmental food cues in confronting individuals with the decision of whether to resist or succumb to the desire to eat (Cohen, 2008). In brief, feeding behaviour is influenced by implicit and explicit factors that together, comprise the main components of reward (liking, wanting, learning) (Berridge and Kringelbach, 2011) (Fig. 1.7).

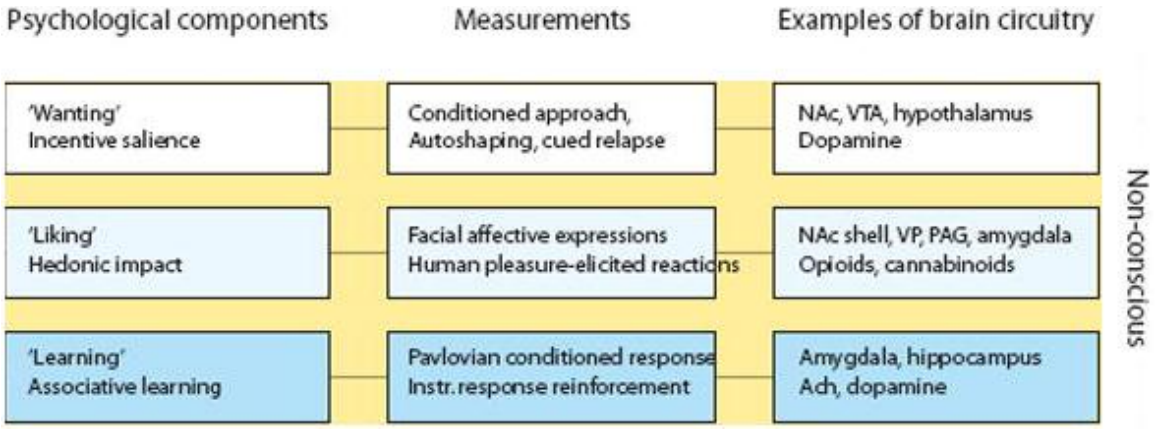

Figure 1.7 The three components of reward and feeding behaviour. Major processes within reward consist of wanting or incentive salience (white), pleasure liking or hedonic impact (light blue) and learning (blue). Each of these contains explicit and implicit psychological components that constantly interact. The non-conscious components, their measurements or behavioural procedures and some of the brain circuitry sensitive to each of these processes are depicted. NAc, nucleus accumbens; VTA, ventral tegmental area; VP, ventral pallidum; Ach, acetylcholine. (*modified from: Berridge and Kringelbach, 2011*).

1.5 The concomitant rise of overweight/obesity and chronic diseases

The various influences on food availability and promotion in modern societies is marked by a general change in eating patterns, in particular the consumption of food in excess of their metabolic needs (Rolls, 2003). Today, eating for pleasure rather than to fulfill need is common and is often coupled with lower levels of activity (labor or recreational). Thus, balancing energy intake and expenditure has not kept pace with this new turn in evolution, the result being overweight and obesity which is reaching epidemic proportions.

At the beginning of the 21st century, "the human race reached a sort of historical landmark, when for the first time in human evolution the number of adults with excess weight surpassed the number of those who were underweight" (Gardner and Halweil, 2000; Levitsky and Pacanowski, 2012). Obesity was formally recognized as a global epidemic by the World Health Organization (WHO) in 1997. The occurrence of obesity nearly doubled since 1980; WHO estimates in 2008 and 2011 were that there are at least 1.4 billion overweight adults, of them 500 million are obese and 40 million overweight. Alarmingly, "65% of the world's population live in countries where overweight and obesity kills more people than underweight" (WHO, 2008).

Obesity is defined as an excess in body fat (adipose tissue) as a result of accumulation of visceral and subcutaneous fat; importantly, however, it is the depot of white adipose tissue, rather than subcutaneous fat, that is considered risky and/or harmful to health (see below). Obesity represents a serious health problem because it diminishes life expectancy, and threatens mental and physical health (Must et al., 1999; Haslam and James, 2005; Scott et al., 2008). The most serious chronic diseases associated with overweight and obesity are type 2 diabetes, coronary heart disease, cancer (especially of the breast and colon), osteoarthritis and hypertension, some of which group under the term "metabolic syndrome" (Lapidus et al., 1994; Brown et al., 2000; Danaei et al., 2011; Wormser et al., 2011; Aballay et al., 2013). There is now strong clinical evidence that obesity compromises brain health and function. For example, depression is a significant comorbidity of severe obesity (Dixon et al., 2003; Moussavi et al., 2007; Hrabosky and Thomas, 2008) and antidepressants tend to be less efficacious in depressed patients who are obese (Kloiber et al., 2007). In addition, increased total body fat (irrespective of distribution) is associated with cognitive impairments (learning,

memory, executive functioning) (Elias MF et al., 2003) and higher risk for developing dementia and Alzheimer disease (Whitmer et al., 2007; Fitzpatrick et al., 2009).

In brief, understanding the biological and behavioural factors that lead to obesity is a major priority for public health.

1.6 Problems addressed in this thesis

Does food addiction cause obesity?

The hypothesis that obesity reflects food addiction gained momentum in the last 15 years, leading to obesity being proposed as a mental disorder in the 5th edition of the American Psychiatric Association's Diagnostic and Statistical Manual (DSM-V), rather than a metabolic disorder (Volkow and O'Brien, 2007). However, the exact description of this disorder is far from settled and many scientists refute the theory. Much of the evidence put forward by the theory's proponents centers around observations of down-regulated striatal dopamine receptor 2 (DR2) in obese persons and cocaine addicts; the authors (Wang et al., 2001) thus concluded that individuals who consume higher amounts of food and drugs do so to compensate for their reduced reward sensitivity (Wang et al., 2001). Other findings supporting the food addiction theory included data showing overlap in the neurochemical mechanisms triggered by food and drugs of abuse. For example, the increase in dopamine release and dopamine receptor activation in corticolimbic brain regions during anticipation and consumption of food (Hernandez and Hoebel, 1988; Small et al., 2001, 2003; Liang et al., 2006) has been likened to the neurochemical profile of subjects showing a preference for substances and drugs of abuse (Wise, 1987; Di Chiara and Imperato, 1988). Other evidence includes reports that rats binge on sucrose and develop addiction-like behaviour when placed under a regimen that involves exposure of hungry animals to intermittently available sucrose (Avena et al., 2005). Further, animals allowed to binge on sugar on a daily basis led to an increase in dopamine release (Rada et al., 2005), increased dopamine transporter expression (Bello et al., 2003) and altered availability of DR2 in the N.acc (Bello et al., 2002), as well as

withdrawal symptoms (resembling opioid withdrawal) after cessation of the daily presentation of sucrose and, cross-sensitization to amphetamine-induced locomotor activity (Avena et al., 2008).

On the other hand, other investigators argued against the above evidence as explanations for overeating and obesity (Epstein and Shaham, 2010; Peters, 2011b; Ziauddeen et al., 2012). The main arguments against the food addiction theory are: i) sugar addiction cannot by itself explain overweight and obesity since the rats that developed this phenomenon did not gain more weight than control animals (Avena et al., 2005); ii) sugar addiction has been only demonstrated in rodents but not in humans (Benton, 2010); iii) empirical data from human subjects showed that not all the individuals that have binge-eating disorders (BED) are obese and only a small percentage of obese individuals have a BED (Wonderlich et al., 2009).

In attempts to untangle the causes of compulsive behaviours like overeating, some authors reported that rats, like many other species, develop three different conditioned approach responses in the pavlovian conditioning paradigm when a reward (food or drugs) is used to reinforce and motivate the behaviour: the different conditioned responses (CR) observed are sign-tracking (ST) conditioned responses, goal-tracking (GT), or intermediate tracking (IT) CRs (Meyer et al., 2012; Tomie et al., 2012). Most authors consider that ST animals will be more vulnerable to develop compulsive behaviours such as addiction and overeating. The animals attribute greater incentive salience to drug or food cues that are predictive of reward (Flagel et al., 2009). Indeed, ST animals were shown to display higher cocaine-seeking behaviour and greater risk for relapse when faced with cocaine-associated cues (Saunders and Robinson, 2010) and, are more sensitive to the psychomotor-stimulating effects of cocaine (Flagel et al., 2008) and alcohol (Tomie et al., 2008). The vulnerability of ST subjects to addictive behaviour has been attributed to higher levels of DR1 dopaminergic receptors in the ventral tegmental area and lower levels of DR2 receptors in the N.acc (Flagel et al., 2007). Consistently, dopamine receptor blockade in the core of the N.acc abolishes ST (Saunders and Robinson, 2012). Together, these observations support the view that ST in the pavlovian conditioning paradigm may predict predisposition to compulsive behaviours.

While many studies (Flagel et al., 2008; Tomie et al., 2008; Saunders and Robinson, 2010) have shown that ST may sign vulnerability to develop addiction to drugs of abuse, there is no evidence that ST is a biomarker of vulnerability to overeat. **The predictive value of ST behaviour in**

overeating is addressed in Chapter 2 of this dissertation and published in *Translational Psychiatry*. Specifically, we aimed to address the following issues:

- Is sign tracking in pavlovian conditioning a predictor of vulnerability to emotional/motivational disturbance and to overeating?
- Do obese mice display addiction-like behaviours?
- Are drug addiction and overeating two different states?

Influence of obesity on cognition and emotion: from humans to animal models

Besides being a risk for physical health, obesity can also affect brain processes and structures involved in reward, learning, and emotional control. Obese individuals appear to be hyper-responsive to environmental cues related to palatable foods, most likely due to enhanced activation of the reward circuitry and emotional centers (Davis et al., 2004; Rothemund et al., 2007). In fact, after exposing overweight and obese children to visual food-related cues, they tend to consume foods in an exaggerated manner, as compared to lean subjects (Jansen et al., 2003; Halford et al., 2008). Conversely, some obese human subjects are hypo-responsive to food ingestion, an effect attributed to reduced striatal responses (Stice et al., 2008). The latter finding is supported by data showing that obese rodents exhibit lower basal levels of dopamine (Geiger et al., 2009), reduced dopamine DA turnover (Davis et al., 2008) and fewer DR2 receptors (Hajnal et al., 2008) in the mesolimbic pathway. These sets of data have been interpreted as reflections of compensatory mechanisms for reward deficiency in obese individuals (Wang et al., 2004).

Many investigators have described associations between high body mass and cognitive function in humans and rodents, although there are many inconsistencies in the reports from individual studies. While some human longitudinal studies report a correlation between higher adiposity and poor performance in global cognitive function, memory and language (Elias et al., 2003; Whitmer et al., 2005; Gunstad et al., 2010), others have reported that higher body mass index (BMI) associates with good cognitive performance, and that weight loss in old age may reduce functional abilities and may represent higher risk for developing Alzheimer disease (Barrett-Connor et al., 1996; Stewart et al. 2005). Similar discrepancies arise in the literature on rodents. While some authors found that

prolonged exposure to obesogenic diets impairs spatial learning and memory in mice (Farr et al., 2008; Morrison et al., 2010) and rats (Molteni et al., 2002; Wu et al., 2004; Jurdak et al., 2008; Stranahan et al., 2008), others failed to observe a negative relationship between obesity and reduced spatial learning in the Morris water maze (Mielke et al., 2006; McNeilly et al., 2011; Pancani et al., 2013). Other studies reported that obese animals have impairments of spatial and working memory in the radial arm maze (Greenwood and Winocur, 1990; Murray et al., 2009; Valladolid-Acebes et al., 2011) and of procedural learning in the operant food paradigm (Greenwood and Winocur, 1990; Mielke et al., 2006; Farr et al., 2008; McNeilly et al., 2011). In most of these experiments, palatable foods were used as the reinforcing stimulus to motivate learning of these paradigms.

The experiments reported in Chapter 3 of this thesis consider that food may not be an appropriate reinforcer in all learning tasks or in testing reward sensitivity in (obese) subjects with abundant energy reserves. The reasoning is that since obese animals will likely have lower energy demands, diminished food consumption in learning paradigms might be falsely interpreted as cognitive and/or reward deficits, rather than a motivational problem. Our specific aims here were to gain insights into the following question:

- Do the lower energetic needs of obese mice mask their ability to evaluate hedonic stimuli and learning potential in appetitive conditioning tasks?

The work presented in Chapter 3 has been submitted for consideration by *Frontiers in Behavioral Neuroscience*.

Feeding behaviour during ageing

Humans are living longer than ever due to progress in medicine (especially hygiene) and science and technology. Longevity has brought other challenges for Society, in particular, ensuring that longer life is accompanied by better quality of health and well-being. Feeding behaviour and caloric intake (as well as physical activity) changes dynamically throughout the lifespan (Fitzgibbon et al., 2000; Okosun et al., 2000; Pescatello et al., 2000). Ageing humans tend to have greater adiposity and, as a result are at greater risk for developing metabolic and cardiovascular disorders which, in turn, predispose them to psychopathologies such as depression and dementia (Elias et al., 2003; Mathus-Vliegen et al., 2005;

Fitzpatrick et al., 2009; Gunstad et al., 2010; Topic et al., 2013). Thus, understanding the neurobiology of feeding in aged subjects is of paramount importance. For example, little is known about whether altered feeding habits during ageing reflect changes in sensory processing or cognitive abilities; and, in contrast to the situation in children and adolescents (Halford et al., 2004, 2008; Boyland et al., 2011), there is sparse information about how environmental food cues (including media proportions) influence eating choices in ageing subjects.

The studies described in Chapter 4 examine food preference and reward responsiveness, motivated behaviour, and appetitive associative learning in mice as a first step towards understanding how eating behaviour is regulated in the ageing human. The specific question asked was:

- Do healthy ageing mice show deficits in associative learning, motivation and hedonic preference for food?

The results are reported in a manuscript currently being evaluated by *Frontiers in Aging Neuroscience.*

CHAPTER 2

Pavlovian conditioning and cross-sensitization studies raise challenges to the hypothesis that overeating is an addictive behaviour

Adapted from:

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2.1 ABSTRACT

Elevated glucocorticoid levels and sign tracking (ST) in pavlovian conditioning are potential biomarkers of compulsive behaviours such as addiction. Since overeating is sometimes viewed as a form of addictive behaviour, we hypothesized that murine pavlovian sign trackers would have a greater propensity to overeat and develop obesity. Using a food reward in the classical conditioning paradigm, we show that ST behaviour is a robust conditioned response but not a predictor of eating and growth trajectories in mice, thus challenging the view that the development of obesity and drug addiction depend on identical mechanisms. This interpretation was supported by experiments which showed overweight mice do not display cross-sensitization to an addictive drug (morphine), and conversely, that overweight morphine-sensitized animals do not over-consume a highly rewarding food. Although the rewarding/motivational effects of both food and drugs of abuse are mediated by similar neurochemical mechanisms, obesity and drug addiction represent a summation of other dysfunctional input and output pathways that lead to the emergence of two distinct disorders, each of which would deserve a specific pharmacotherapeutic approach.

2.2 INTRODUCTION

Overweight and obesity may be direct or indirect antecedents of neuropsychiatric disorders such as depression, anxiety, stroke and dementia (Scott et al., 2008; Kivimäki et al., 2009) on the other hand, psychiatric conditions and/or certain psychotropic medications may lead to overweight (Allison et al., 2009). Associative learning plays an important role in eating behaviour and is particularly important in the context of the current obesity epidemic because of the impact of peer pressure and advertising on the formation of eating habits (Cohen, 2008). Pavlovian (classical) conditioning is a simple form of appetitive learning where individuals develop conditioned responses to external cues; over time, the brain implicitly attributes high motivational valence to stimuli that previously carried no value. Although an evolutionarily conserved form of learning that facilitates adaptation, conditioning may underpin maladaptive behaviours, including overeating and obesity (Martin-Soelch et al., 2007; Saunders and Robinson, 2013). Learned cues can result in overeating by overriding satiety signals (Weingarten, 1983; Jansen et al., 2003; Petrovich et al., 2007b; Halford et al., 2008) through the activation of brain circuits involved in sensory processing and reward anticipation as well as emotional centers involved in memory and habit formation (Rothemund et al., 2007).

There are three types of conditioned responses (CR): sign-tracking (ST), goal tracking (GT) and intermediate tracking (IT, alternate between the location of reward delivery (US) and the conditioned stimulus (CS+)). In contrast to GT, ST subjects make more approaches to the CS+ *vs.* unconditioned (US) stimulus; their "cue reactive" behaviour is thought to result from attribution of "incentive salience to reward cues, transforming predictive conditional stimuli to incentive stimuli with powerful motivational properties" (Saunders and Robinson, 2012). Research in animals suggests that ST reflects impairments in behavioural inhibition and vulnerability to drugs and substances of abuse (Flagel et al., 2008; Tomie et al., 2008; Saunders and Robinson, 2010).

Overeating in excess of physiological need has been likened to other compulsive (addictive) behaviours, in particular because anticipation and consumption of food increases dopamine release and dopamine receptor activation in the corticolimbic brain (Hernandez and Hoebel, 1988; Small et al., 2003), resembling the neurochemical profile of subjects showing a preference for drugs of abuse (Di Chiara and Imperato, 1988). This overlapping has triggered a lively debate about whether overeating and obesity represent an addictive behaviour (Avena et al., 2008, 2012; Epstein and Shaham, 2010; Johnson and Kenny, 2010; Peters, 2011b; Ziauddeen et al., 2012; Volkow et al., 2013; Ziauddeen and

Fletcher, 2013) a core tenet of the "food addiction hypothesis" is based on the fact that dopaminergic circuits, involved in motivation and reward, are activated in obese and drug addicted states (Volkow et al., 2013). Other support for the "addiction hypothesis of obesity" derived from studies in which binging on sugar by rats was interpreted as "sugar addiction" (Avena et al., 2008, 2012). On the other hand, the weaknesses of the experimental paradigms used and the limitations of extrapolating the findings in rodents to humans have been discussed in an instructive review (Ziauddeen and Fletcher, 2013). The present study approached the question from a different perspective, using the pavlovian conditioning paradigm. Our results show that ST behaviour is not associated with greater consumption of highly rewarding foods and that there is no cross-sensitization between food and the highly addictive psychoactive drug (morphine).

2.3 METHODS

Animals: Experiments conformed to local and national ethical guidelines, including the precepts of European Union Directive 2010/63/EU. Male C57BL6 mice (Charles River, Sulzfeld, Germany), aged 3-4 months, were used; mice were housed in pairs under standard laboratory conditions. All diets were from Charles River. Behavioural tests were conducted during the animals' activity phase after 1 week of habituation (room, experimenter, calorie-restriction schedule to induce 10-15% loss of original body weight); unless otherwise stated, the calorie restriction schedule was applied throughout; mice had *ad libitum* access to water. In some experiments, variable degrees of overweight were induced by maintaining mice on either a standard laboratory (normal) chow (NC), low-fat diet (LFD, # D12450B; 16.1 J/g, 10% from fat, 70% from carbohydrate) or a high- fat diet (HFD, D12451; 19.8 J/g, 45% from fat, 35% from carbohydrate). Diet-induced obesity was induced over 3 months by maintaining animals on HFD.

Pavlovian conditioning: Autoshaping was performed in automated touchscreen chambers, as previously described (Horner et al., 2013). The conditioned stimulus (CS) was a 10 s flash of white light in either the left- (50% of animals) or right- (50% of animals) hand side of the screen. Immediately after stimulus offset, a liquid food reward (15 µl of diluted condensed milk [14% sugar], low-fat cream [5% fat, 8.7 kJ/g] or high-fat cream [32% fat, 13 kJ/g]) was delivered into the food magazine.

During task acquisition mice were trained to associate the light stimulus (CS+) with reward delivery. During each session (1/day), presentations of 15 CS+ and 15 CS- were made in a randomized order (maximum of 2 consecutive presentations of same stimulus, VI schedule of 10-40 s between each stimulus). Animals reaching criterion (70% of correct [CS+] approach responses/session on at least 3 consecutive days) were designated as ST. Retention was tested 2 weeks after the acquisition phase (all test conditions as during acquisition phase) in satiated animals (3 consecutive days of food *ad libitum*). Extinction of conditioned responses (CR) commenced 1 d after completion of retention testing. All test parameters were as before except that approaches to the CS+ were not rewarded, and training sessions were conducted until mice made an equal number of CS+ (15) and CS- (15) approaches over at least 2 consecutive sessions. In each session, (i) number of CS+ and CS-

approaches, (ii) mean latency to approach CS+ and CS-, (iii) mean latency to collect food reward following correct responses and, (iv) session completion time, were recorded.

Test of motivational state: This test (independent of learning strategies) was carried out over 2 d in the touchscreen chambers (reward: 15 µl of milk containing 14% sugar). The latency to retrieve all of the reward and number of food tray entries were monitored in each session (15 reward cycles, delivered with a VI of 10-40 s).

Tests of emotionality: All tests were performed during the daily phase of activity (lights off in animal housing room). The open field (OF) test was used to measure locomotor activity and explorative behaviour, 4 weeks after autoshaping; animals were housed as before, with food and water available *ad libitum*. The open field arena was made of Plexiglass (white base: 30 X 30 cm; dark grey walls: 30 cm high). Testing was done in a dark room but the arena was uniformly illuminated with white light (100 lux). Activity of the mice was recorded using a video camera and the results were subsequently analyzed using ANY-maze software (Stoelting, Wood Dale, IL). Mice were tested in the apparatus for 5 min, and the total distance travelled and time spent in the center was computed for each mouse.

Mice were subsequently habituated to the OF apparatus over 2 more days before being subjected to a novel object (NO) test to examine reactivity to novelty. The novel object was a small plastic toy placed in the center of the OF; video-tracking with Any-maze software was used to measure interaction times with this unfamiliar object.

Stress-coping behaviour was analyzed in a 1-session version of the forced-swim test (FST) by monitoring floating vs. swimming time (higher floating time indicated better stress-coping strategy). The FST apparatus consisted of an acrylic glass cylinder (dimensions: height x radius: 60 x 15 cm) filled with tap water (25° C); the water was changed between every trial. Animals were placed in the cylinder for a total of 6 min, but behaviour was recorded during the last 4 min only. Testing was carried out in a dark room, but the FST cylinder was directly illuminated with white light (80-100 lux). Floating and swimming times were recorded by video camera and videos were analyzed with Any-maze software. Mice that were immobile, with movements of only the hind legs to maintain balance, were considered

to be floating; use of tail movements to maintain the head above water was scored as swimming behaviour.

Neuroendocrine response to stress: The dynamic reaction of the hypothalamo-pituitary-adrenal (HPA) axis to an acute stress (vortexing in 200 ml glass beaker, 2 min) was evaluated by measuring blood corticosterone (125I-Corticosterone RIA kit, ICN Biochemicals, Costa Mesa, CA) in ventral tail vein samples collected at intervals for up to 120 min.

Morphine cross-sensitization in mice of differing body masses: Mice maintained on either a standard laboratory diet, LFD or HFD were subjected to a slightly modified version of a previously published cross-sensitization protocol (Salomon et al., 2006), schematized in Fig. 2.5A.

Sucrose consumption test: Mice that had been satiated on either LFD or HFD were given a choice between water and a 5% sucrose drinking solutions. Fluid consumption of which was measured at 3, 6 and 24 h thereafter; importantly, food was available *ad libitum* throughout, allowing assessment of the hedonic value of the highly-rewarding sucrose solution, independently of the animal's state of satiety or energetic needs. Immediately thereafter, animals were food-deprived for 24 h and provided the water-sucrose choice, allowing discrimination between sucrose consumption due to hedonic liking vs. energetic needs.

Data analysis: Statistical analysis was performed with Prism 5.0 statistical package (GraphPad, La Jolla, CA). Data were first subjected to 1- or 2-way ANOVA, followed by Bonferroni-corrected post-test comparisons. The level of significance was set at $p<0.05$.

2.4 RESULTS

Divergent conditioned responses do not imply differences in learning ability

Associative learning plays an important role in the shaping of eating and other behaviours. After conditioning, the brain implicitly attributes high motivational valence to a previously neutral stimulus. Here, calorie-restricted male mice were rewarded with a liquid food (sweetened milk) in the pavlovian conditioning paradigm in which light served as the neutral stimulus. Based on their conditioned responses (CR) on the last 3 days of conditioning, mice were categorized as sign trackers (ST, minimum 65% approaches to CS+), goal trackers (GT, less than 20% approaches to CS+), or intermediate trackers (IT, 20-65% approaches to CS+).

None of the mice differed in learning ability, as judged by time to complete the sessions (Fig. 2.1.1A) or reward retrieval latency (Fig. 2.1.1B). Notably, all animals required progressively shorter times to complete the task [$F_{10,303}$=26.5;$P \leq 0.0001$] (Fig. 2.1.1A). Absence of learning impairments was further confirmed by data on the relative number of approaches (Fig. 2.1.2A) and total number of approaches (Supplementary Fig. 2.1.2B) towards the CS-, as well as the latency to approach the CS- (Fig. 2.1.2C). Interestingly, 42, 35 and 23% of the mice showed segregation into ST, GT and IT behaviours, respectively, [$F_{2,303}$=409.8;P<0.0001] (Fig. 2.1.1C). The ST and GT animals consistently showed significant differences between sessions 3 and 11 ($P \leq 0.001$) and, while the ST group made progressively more CS+ approaches, the number of CS+ approaches by GT animals steadily declined over successive test sessions. In contrast to ST and GT mice, IT mice alternated between the CS+ and CS- with similar frequencies during sessions 3-11 ($P \leq 0.001$ *vs.* ST and GT groups) (Fig. 2.1.1C). Overall, significant differences were also seen in the total number of CS+ approaches [$F_{10,303}$=4.6;$P \leq 0.0001$] and all groups differed from one another in terms of overall CS+ approaches [$F_{2,303}$=51.2;$P \leq 0.0001$] (Fig. 2.1.2D). Lastly, ST, GT and IT differed significantly in their latencies to approach the CS+ [$F_{2,300}$=138.61;$P \leq 0.0001$] (Fig. 2.1.1D); notably, compared to ST animals, GT animals showed higher latencies to approach CS+ ($P<0.001$) (Fig. 2.1.1D).

Strength of conditioning was demonstrated by testing retention in a separate set of ST animals. As shown in Fig. 2.1.1E, the relative number of CS+ and CS- approaches on the first day of testing (session 1 in *second-from-left panel* of Fig. 2.1.1E) did not differ significantly from that observed in the last session (session 11 in *left-hand panel* of Fig. 2.1.1E); importantly, however, ST mice approached the CS+ more than CS- [$F_{1,48}$=167.1;$P \leq 0.0001$]. Further, the same retention patterns were observed

when, animals were tested for their CS approaches when satiated (i.e. 3 d food *ad libitum*); as shown in Fig. 2.1.1E (*third panel from left*), the relative number of CS+ *vs.* CS- approaches were significantly different [$F_{1,48}$=223.1;P≤0.0001]. Next, we asked if the conditioned memory could be extinguished by reward non-delivery. As depicted in Fig. 2.1.1E (*right-hand panel*), ST mice continued to make more CS+ than CS- approaches [$F_{1,120}$=276.8;P≤0.0001] during all 10 sessions.

Briefly, this set of experiments demonstrates that mice adopt ST, GT or IT behaviours (CR) which, nevertheless, do not reflect differences in learning ability. Moreover, the CR of ST mice to a food reward are robustly retained, hard to extinguish, and persist even when the animals are food-satiated.

Segregated learning curves persist independently of reward value

Learning is strongly influenced by motivational state (Jarvandi et al., 2009; Olausson et al., 2013). The latter increases as the subjective or real value of a reward increases and sweet and fatty foods carry particularly high incentive salience for many species, including mice (Sclafani, 2004).

Mice were assigned to receive either a low-fat (5%, n=24) or high-fat (32%, n=22) reward during pavlovian conditioning. By clustering animals according to their acquisition of the task we observed segregation of animals into ST, GT and IT groups, irrespective of the conditioning reward (Fig. 2.2A,B) (High-fat reward: $F_{2,209}$=248.9;P≤0.0001; Low-fat reward: $F_{2,231}$=238.9;P≤0.0001). Reward value (high-fat vs. low-fat) did not alter the rate of CR acquisition by ST (Fig. 2.2C) and GT (Fig.2D) mice, with ST and GT animals respectively displaying gradual increases [$F_{10,165}$=22.4;P≤0.0001] and decreases [$F_{10,143}$=6.02;P≤0.0001] in approaches to the CS+ over time. As expected, IT mice fluctuated between CS+ and CS-, irrespective of the value of the reward (Fig. 2.2E). Thus, individual expressions of ST, GT and IT conditioned responses evolve independently of reward value.

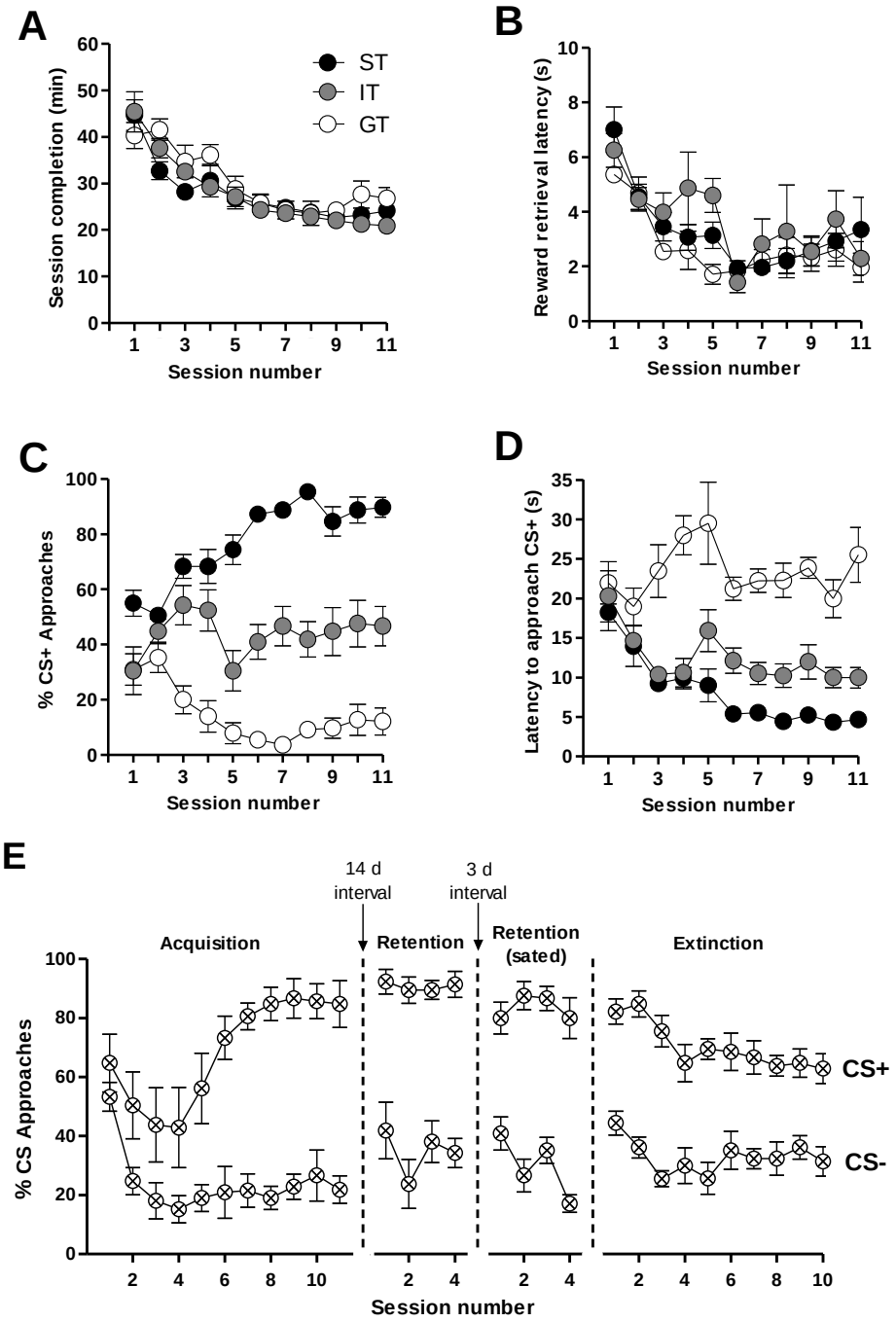

Figure 2.1.1 Acquisition of conditioned responses. Mice displayed different conditioned responses, sign-tracking (ST, predominantly approached the CS+; n = 13), goal-tracking (GT, predominantly approached the US; n = 11) and intermediate-tracking (IT, alternated between CS+ and US with approximately equal frequency; n = 7). Autoshaping was monitored over 11 sessions; in each session, mice received 15 CS+ and 15 CS- presentations. **A.** Time (min) for completion of session. **B.** Mean latency (s) to retrieve the food reward. **C.** Relative number of CS+ approaches during each session. **D.** Mean latency (s) to approach the CS+ during each session. **E.** Results derive from a cohort of ST mice (n = 7) different to that used in upper panels (A-D). *Left-most panel* shows acquisition of the task (11 sessions). After a 14 d interval, mice were tested for retention of their conditioned responses under standard testing conditions involving food restriction (*second-from-left panel*) and, following a 3 d interval, retention was tested when mice were fed *ad libitum* (*second-from-left panel*). The *left-most panel* depicts results of an experiment to test extinction of the conditioned response; over 10 consecutive sessions. Data shown are means ± SEM.

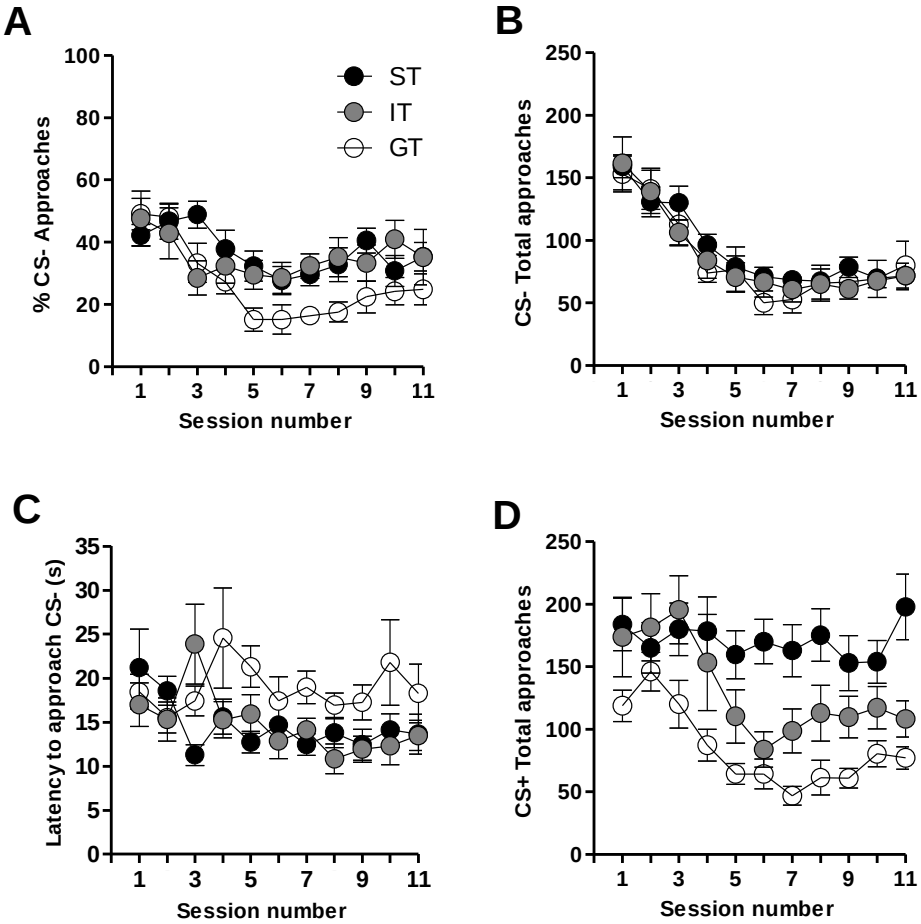

Figure 2.1.2 Acquisition of conditioned responses. Mice displayed different conditioned responses: sign-tracking (ST, predominantly approached the CS+, n = 13), goal-tracking (GT, predominantly approached the US, n = 11) and intermediate-tracking (IT, alternated between CS+ and CS- with approximately equal frequency, n = 7). Autoshaping was monitored over 11 sessions; in each session, mice received 15 CS+ and 15 CS- presentations. For each session, the relative number of CS- approaches **(A)**, total number of CS- approaches **(B)**, mean latency (s) to approach the CS- **(C)** and total number of CS+ approaches **(D)**, are shown. Data represent means ± SEM.

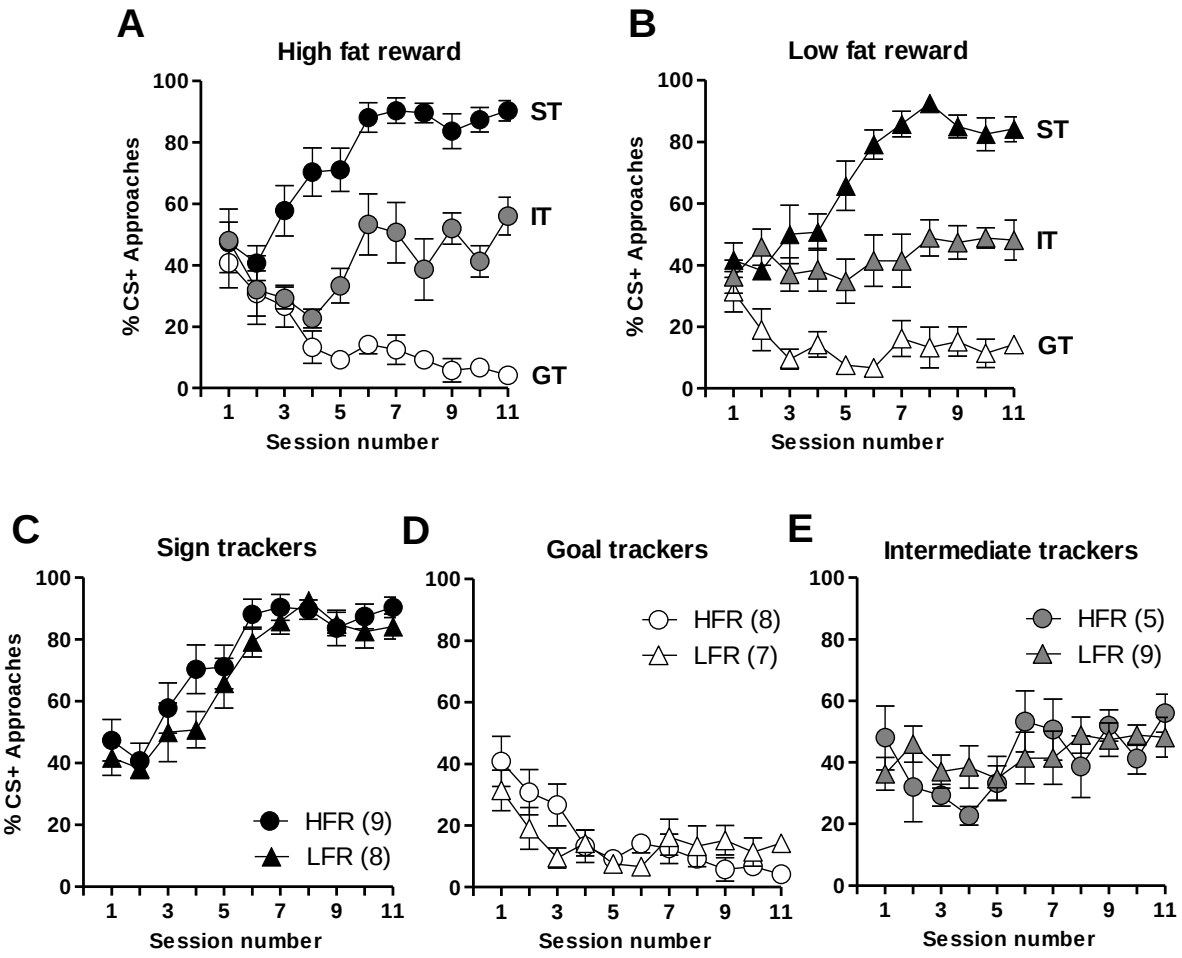

Figure 2.2 Conditioned responses do not shift with changes in reward value. Shown are the approaches to the CS+ in each of the 11 test sessions consisting of 15 CS+ and 15 CS- presentations. **A.** Mice rewarded with a high-fat reward segregated into sign-trackers (ST; n = 9), goal-trackers (GT; n = 8) and intermediate trackers (IT; n = 5). **B.** Mice rewarded with a low fat liquid reward segregated into sign-trackers (ST; n = 8), goal-trackers (GT; n = 7) and intermediate trackers (IT; n = 9). Acquisition of ST, GT and IT conditioned responses to high-fat or low-fat rewards is shown in **C, D** and **E**, respectively. Data are presented as means ± SEM.

Sign tracking does not predict impaired emotionality or responses to stress

Associations between sign-tracking behaviour, susceptibility to addictive behaviours and, exaggerated corticosterone responses to stress and hyper-emotionality were reported previously (Flagel et al., 2010). Sign-, goal- and intermediate-tracking CR are commonly observed in pavlovian conditioning (Hearst and Jenkins, 1974; Boakes, 1977) and sign-tracking, in association with hyperemotionality and exaggerated responses to stress, is suggested to presage addictive behaviour (Saunders and Robinson, 2013). We here asked, Is sign tracking behaviour *per se* a general predictor of dysregulated behaviour?

Previously-designated ST, GT and IT mice (Fig. 2.1.1) showed similar baseline levels of corticosterone (Fig. 2.3A) and responded to a brief stressor with increased corticosterone secretion within 30 min [$F_{2,81}$=53.7;$P\leq$0.0001] (Fig. 2.3A); however, ST mice displayed a more robust endocrine response to stress than GT and IT mice ($p<0.05$), suggesting that they are more reactive to stress. On the other hand, corticosterone levels returned to baseline in all groups by 120 min after the stress, indicating intactness of corticosterone negative feedback regulatory mechanisms in ST animals.

High stress reactivity is linked to compromised coping behaviour in unfamiliar or hostile environments (Sousa et al., 2006). Here, ST, GT and IT mice did not show differences in emotionality or stress-coping behaviour between ST, GT and IT mice, as measured by locomotor activity, interaction with a novel object and, struggling *vs.* floating times in a forced-swim test (Fig. 2.3B-E).

In summary, the display of high stress reactivity by ST mice is not predictive of increased emotionality, a factor thought to contribute to increased vulnerability to addictive behaviours.

Motivational behaviour is intact in sign-trackers

Sign tracking rats show alterations in mesolimbic dopaminergic transmission (Flagel et al., 2007), reflecting altered motivational state. Here, application of food retrieval test in calorie-restricted animals in order to assess behavioural characteristics that would inform on motivation showed that ST, GT and IT retrieved a highly rewarding food (sweetened milk) with similar latencies (Fig. 2.3F), consumed the reward in a similar time (Fig. 2.3G) and made a similar number of food-tray entries (Fig. 2.3H). These findings indicate that, although ST conditioned responses to cues predictive of food reward are associated with higher vulnerability to compulsive behaviours like addiction (see Fineberg et al., 2010), ST *per se* does not equate to increased motivational drive for food reward.

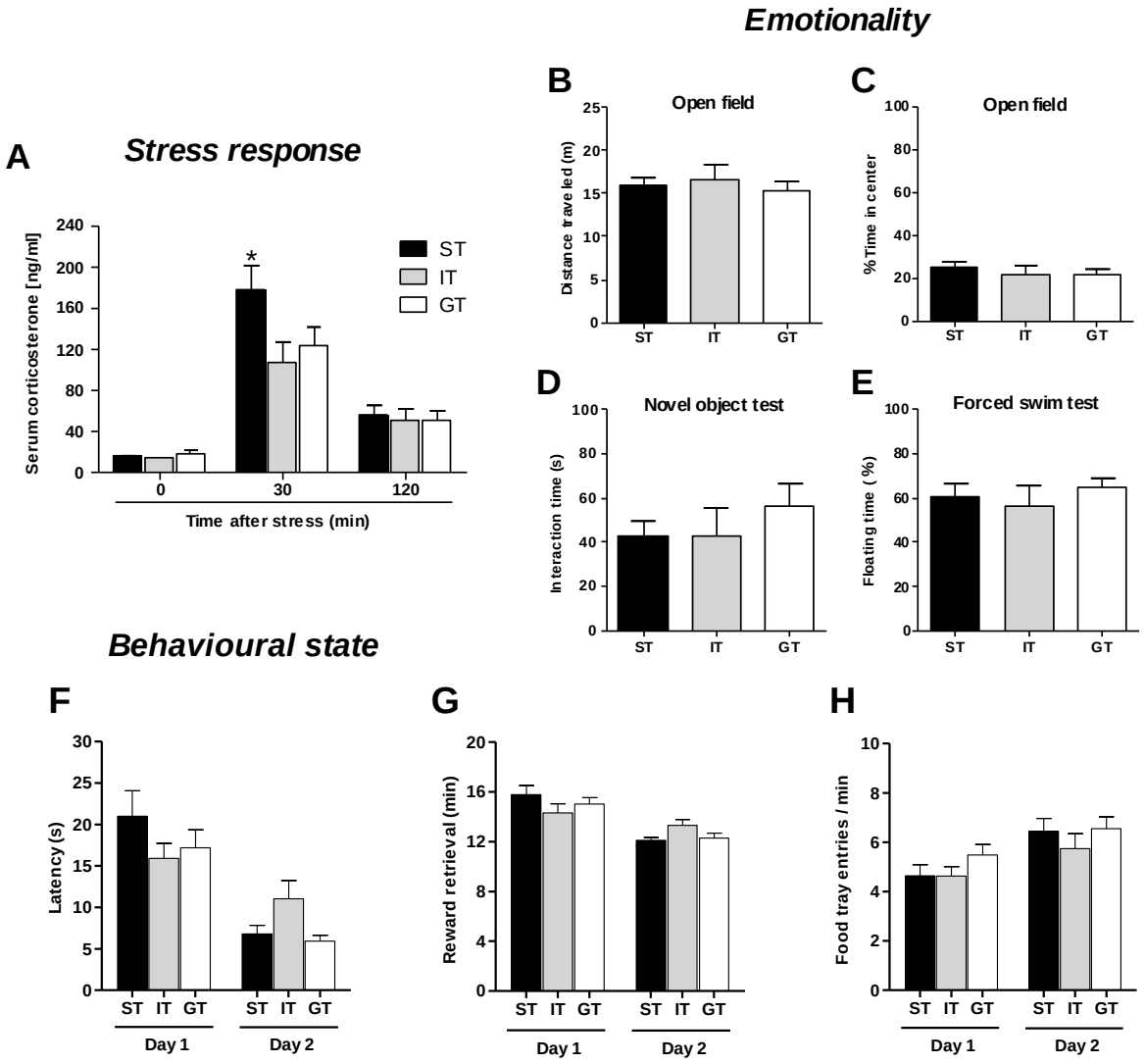

Figure 2.3 (A-D) Stress-coping in and emotional phenotype in sign-tracking (ST), goal-tracking (GT) and intermediate-tracking (IT) mice. Tests were performed in ST (n = 13), GT (n = 11) and IT (n = 7) mice. **A.** Serum corticosterone levels mice under basal conditions (*0 min after stress)* and 30 and 120 min following an acute stressor (see *Materials and Methods* for experimental details) are depicted. Note that although ST mice showed the most robust hormonal response to the stressor, all animals had similar levels of corticosterone 120 min post-stress, indicating that glucocorticoid negative feedback mechanisms were unimpaired in the ST group. **B-C.** Locomotor activity was monitored in an open field arena. Two parameters were monitored: total distance travelled (m) and time spent in the center of the arena during a 5 minute test period. **D.** The novel object test was used to assess emotionality in terms of time spent exploring an unfamiliar object placed in the center of an open field arena. Interactions of ST, GT and IT mice with the novel object were monitored over 5 min. **E.** Stress-coping behaviour in ST, GT and IT mice was compared in a 1-session forced-swim test. All groups of mice showed similar times spent floating, i.e. showed identical stress-coping capacities. The depicted data are means ± SEM; the asterisk indicates a higher value in ST ($p < 0.05$), compared to GT and IT. **(F-H) Motivation for food reward does not differ between ST, GT and IT mice.** Animals were rewarded with sweetened milk, considered to be more rewarding than their standard food pellets. Shown are the mean latency to approach the reward **(F)**, the time taken to retrieve (and consume) the food reward **(G)**, and the number of food tray entries **(H)** by ST, GT and IT mice. Measurements were made over 2 sessions, with 15 reward deliveries in each. The results are shown as means ± SEM.

Sign-trackers do not over-consume a highly rewarding food

As mentioned before, rodents prefer fat-rich diets (Sclafani, 2004). Since ST animals are reportedly more vulnerable to compulsive behaviour (Flagel et al., 2009), we here compared the preferences of ST, GT and IT mice for a high-fat diet (HFD) *vs.* low-fat diet (LFD, used to control for novelty of HFD). Daily monitoring of LFD *vs.* HFD food intake over the first 6 days of the experiment revealed that ST, GT and IT animals consumed negligible amounts of the LFD (data not shown), displaying similarly high preference for the HFD (Fig. 2.4A). Over a 3-month exposure to HFD, all groups of mice showed body mass increases [$F_{6,196}$=85.2; $P \leq 0.0001$], albeit to the same extent (Fig. 2.4B). Thus, ST behaviour does not necessarily imply over-eating of rewarding foods and proneness to overweight and/or obesity.

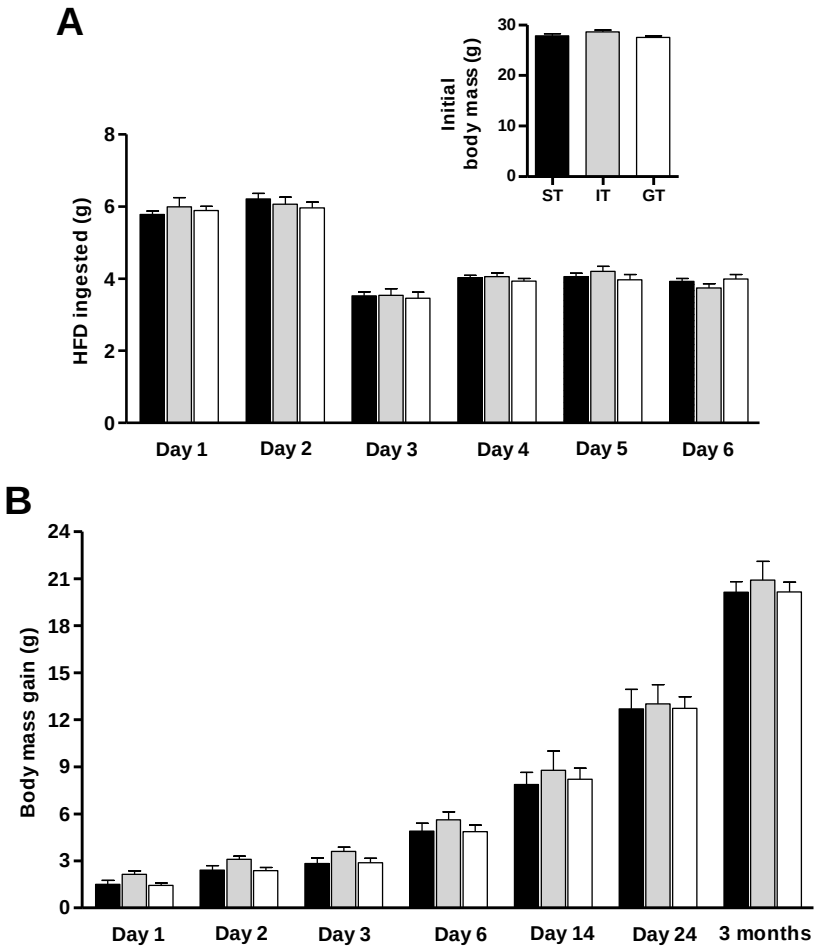

Figure 2.4 Patterns of consumption and body mass growth curves in sign-tracking (ST), goal-tracking (GT) and intermediate-tracking (IT) mice maintained on a high-fat diet (HFD). Sign-trackers (ST; n = 13), goal-trackers (GT; n = 11) and intermediate trackers (IT; n = 7) were placed on a highly palatable high-fat diet, available *ad libitum*, for a period of 3 months. The consumption of the HFD was similar in all groups during the first 6 days of exposure to the HFD **(A)**; note that groups did not differ in their initial body masses (*inset*) and showed similar gains in body mass over the 3 month duration of the experiment **(B)**. The data shown are means ± SEM.

Lack of morphine cross-sensitization in overweight mice

Sensitization to the incentive and motivational properties of drugs of abuse is considered a primary cause of drug addiction (Robinson and Berridge, 2008) and is accompanied by hyper-responsiveness of the mesolimbic reward pathway to dopamine, a feature found in drug addicts and overweight/obese individuals (Volkow et al., 2008b). Accordingly, we used a cross-sensitization paradigm to examine whether mice displaying varying degrees of overweight ($p<0.0001$) would display sensitization (hyperlocomotion) to a single injection of another reward-associated stimulus (morphine). Metabolic status did not influence baseline locomotor activity (10 min in open field after a saline injection) and locomotor activity was equally increased ($p<0.0001$) in all groups after a single injection of morphine (20 mg/kg) (Fig. 2.5A). After a 3-week drug-free period, the same animals received 4 consecutive injections of morphine (20 mg/kg), followed by 4 days of withdrawal from morphine. Similar locomotor activity was displayed by all mice after a final injection of morphine (20 mg/kg; Fig.5A), indicating that, irrespective of their maintenance diets (NC, LFD, HFD) or body mass, all animals were similarly sensitized to the opiate ($p<0.01$, compared to initial acute morphine injection).

Morphine-sensitization does not alter sensitivity to food reward

Since inadequate dopaminergic transmission in the mesolimbic reward pathway appears to be a central mechanism in drug addiction (Robinson and Berridge, 2008), it was of interest to examine whether previous sensitization of overweight mice to an opiate alters sensitivity to a food reward (5% sucrose solution); half of the mice were previously sensitized to morphine. Morphine-sensitized and non-sensitized mice consumed similar amounts of sucrose when satiated ($p>0.05$; Fig. 2.5B), indicating that treatment groups did not differ in terms of reward homeostasis or attribution of hedonic values to sucrose and, that sucrose liking is not a function of satiety level. Indirect evaluation of the influence of energy state on this measure was made by repeating the study in food-deprived (24 h) animals. This experiment revealed that, notwithstanding the possible stress associated with food deprivation, morphine-sensitized and non-sensitized mice ingested similar amounts of sucrose ($p>0.05$; Fig. 2.5C).

Together, these results confirm the intactness of reward mechanisms in mice on diets that differ in reward value; moreover, they demonstrate that previous sensitization to a drug with addictive potential (morphine) does not interfere with the response to a rewarding food.

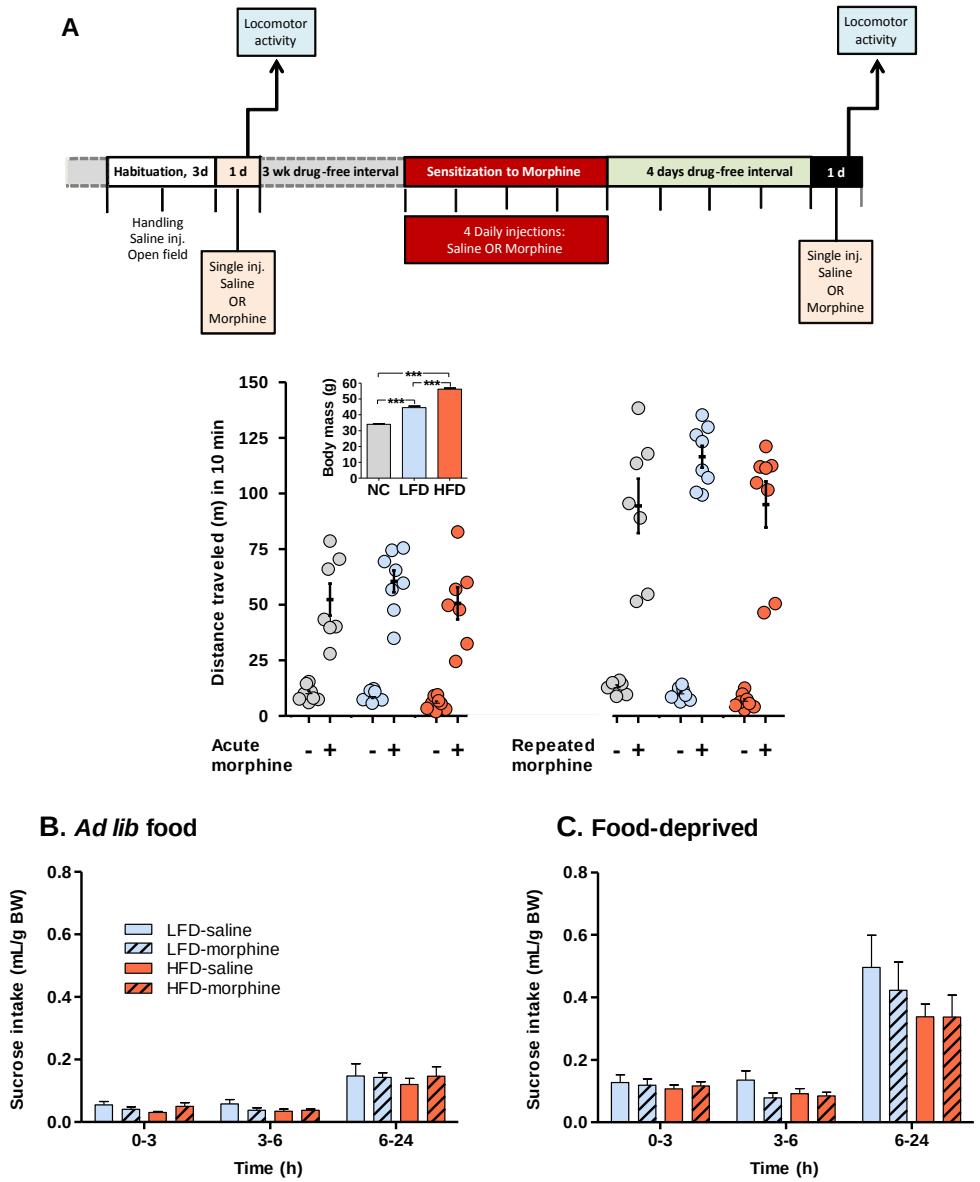

Figure 2.5 (A) Morphine cross-sensitization in overweight mice. The experiment was carried out in mice of varying degrees of overweight/obesity through maintenance on normal chow (NC, n = 15), LFD (n = 16) and HFD (n = 16); the experimental design is shown schematically in the *upper panel*. Initial body masses of the different groups are shown in the *inset*. Animals were habituated over 3 d to the experimental procedure through handling by the experimenter and injection of saline i.p. (0.2 ml); mice were then introduced into the open field (OF) test arena (5 min/d; arena specifications and test conditions are described in legend to Fig. 3). Following habituation, animals received i.p. injections of either saline or morphine (20 mg/kg) and returned to their home cages for 20 min before placement in the OF for 10 min during which time their locomotor activity was recorded. After a 3 week interval, mice were given 4 consecutive i.p. injections of morphine (20 mg/kg/d) or vehicle and kept morphine-free (withdrawn) for 4 d before administration of an acute injection of morphine (20 mg/kg) or saline, after which they were returned to their home cages (20 min) and then monitored for locomotor activity in the OF over 10 min; video recordings of the latter were evaluated using Anymaze software. Results obtained after the acute injections are shown in the *left-hand panel, those after* repeated morphine or saline injections are depicted in the *right-hand panel*. Data are shown as means ± SEM. **(B-C) Sucrose consumption test in morphine-sensitized mice in differing states of obesity.** Experiments were performed in animals that were maintained on either LFD or HFD and some of which were sensitized to morphine (LFD-saline, n = 8; LFD-morphine, n = 8; HFD-saline, n = 7; HFD-morphine, n = 7). Animals were provided with solution of water and 5% sucrose; intake of sucrose was monitored at intervals over a period of 24 h, when mice were satiated (*ad libitum* food) **(B)** or food-deprived for 24 h **(C)**. Depicted data are means ± SEM.

2.5 Discussion

Eating, an essential behaviour, depends on the dynamic integration of potentially conflicting peripheral and cerebral signals. Associative learning, important for acquisition of "liking" of foods, can evolve into conditioned, and eventually uncontrollable "wanting" or "desire"; intense wanting leads to compulsive overeating in excess of physiological needs and eventually, obesity. Since compulsive behaviour is a characteristic of addictive behaviour (Sousa et al., 2006), a popular hypothesis is that excessive eating represents an addictive state (Avena et al., 2008; Johnson and Kenny 2010; Zilberter, 2012; Volkow et al., 2013).

Using pavlovian conditioning, we show that individual mice can form strong associations between a conditioned stimulus and food (cf. Meyer et al., 2012; Tomie et al., 2012). This goal tracking (GT) behaviour contrasts with two other robust types of conditioned responses (CR), namely, intermediate tracking (IT, alternation between conditioned and unconditioned stimuli) and sign tracking (ST, persistent responding to the conditioned stimulus). Importantly, the reward value did not influence the rate of acquisition of these CR patterns, indicating that the associative learning occurred independently of changes in motivational state. This interpretation was supported by data from independent experiments in which all mice made a similar number of food-tray entries and showed similar reward retrieval and consumption latencies. Since the mice used in this work were not selected on the basis of any pre-existing behavioural or physiological traits, the behavioural responses observed cannot be attributed to the nature of the CS and US, but rather reflect natural variations in conditioned learning in general.

Sign tracking behaviour is interesting in the context of our central question: *Can an essential function like eating transform into an addictive behaviour?* Sign trackers are thought to be vulnerable to compulsive behavioural disorders, including addiction, because they "attribute incentive salience to cues that are predictive of reward" (Flagel et al., 2009); this is exemplified by the fact that ST animals display a higher sensitivity to cocaine cues (Saunders and Robinson, 2011) and cocaine-induced hyperlocomotion (Flagel et al., 2008). The vulnerability of ST to addictive behaviour is attributed to altered dopamine receptor expression in the ventral tegmental area-nucleus accumbens motivation-reward circuitry (Flagel et al., 2007) where dopamine receptors play an essential role in the manifestation of ST behaviour (Saunders and Robinson, 2012). Interestingly, obese humans display reduced dopamine binding in the mesocorticolimbic reward pathway (Wang et al., 2001; Volkow et al.,

2008b); this pathway is similarly activated in "food addicted" (obese) and drug addicted subjects (Rothemund et al., 2007; Volkow et al., 2008; Gearhardt et al., 2011). These associations form the backbone of the hypothesis that overeating is a type of addictive behaviour (Volkow et al., 2013).

Excessive eating, especially of energy-dense foods, may be a cause or consequence of stress and the ensuing hypersecretion of glucocorticoids (Born et al., 2010; Groesz et al., 2012; Maniam and Morris, 2012), both potentially important etiopathogenic factors in drug addiction (Sinha and Jastreboff, 2013). While previous authors reported greater sensitivity of rats to the stressful effects of to the stress induced by the autoshaping procedure itself (Flagel et al., 2009; Tomie et al., 2012), rigorous profiling in the present study did not disclose dysregulation of the dynamic regulation of the endocrine response to stress in any of the mice. Novelty-seeking, another correlate of vulnerability to drug abuse, is often associated with hyper-responsiveness, to unfamiliar environments, reflected in parallel increases in glucocorticoid secretion (Piazza et al., 1989, 1993). Interestingly, the latter is associated with impaired mood and affect (Sousa and Almeida, 2012), conditions associated with propensity to self-administer drugs and other substances of abuse (Sinha, 2001). Altogether, the above similarities between ST, IT and GT mice suggest that, unlike the situation in drug addicted subjects (Flagel et al., 2009), susceptibility to overeating is not directly linked to ST behaviour, stress reactivity, stress-coping behaviour or emotionality. It is important to note that ST behaviour is highly predictive of addictive behaviour has been recently challenged by observations that GT animals display two characteristics of vulnerability to addiction, namely, context-conditioned hyperactivity and context-induced reinstatement of drug-seeking behaviour (Robinson et al., 2014).

Given the evidence that ST signifies risk for compulsive behaviour (Fineberg et al., 2010; Yager et al., 2013), it is interesting that our ST, GT and IT mice did not display significantly different body mass gains when given a choice of HFD *vs.* LFD over 3 months. Rather than ascribing this result to long-term homeostatic adjustments in ST animals (all groups ingested similar amounts of the HFD during the introductory phase when factors such as novelty and affect would be expected to play a significant role in shaping subsequent eating behaviour), we suggest that excessive eating is triggered simply by the availability of palatable food and that ST *per se* does not predispose individuals to seek highly-rewarding foods. On the other hand, we cannot rule out i) that HFD, which is more rewarding in terms of sensory stimulation and calorific value, induces liking, wanting and, ultimately, compulsive eating, or ii) that metabolic and other physiological responses to the energetic and other nutritional components of HFD themselves drive eating to match physiological demands.

Like those of drugs and substances of abuse, the rewarding properties of food are mediated by dopaminergic neurons in the mesocorticolimbic pathway (Volkow et al., 2008a). The compulsive consumption of all these rewards appears to result from a hijacking of the homeostatic mechanisms that control motivational status, affect, decision-making and behavioural inhibition (Kringelbach et al., 2012). However, whereas the subjective reward salience of addictive drugs plays an important role during initiation of the addictive process, the reward salience of food is determined not only by its sensory properties but also by the subject's physiological and metabolic status. A tenable alternative to the idea that "food addiction" is responsible for overeating and obesity is expounded in the emerging holistic "hedonic theory of eating" (Berridge and Kringelbach, 2008, 2013) which factors in the important contribution of sensory and peripheral (e.g. energy balance) elements into the complex equation that determines eating and other appetitive behaviours. Specifically, hedonic theory posits that excessive consumption of a particular food results from delivery of specific sensory "pleasure(s)" that override homeostatic "stop eating" signals.

Our initial hypothesis that ST behaviour can be used to identify animals that are susceptible to overeating proved false. To further examine the question of whether overeating is an addictive behaviour, we borrowed the paradigm of behavioural (psychomotor) sensitization from the drug addiction field which considers such sensitization critical for the attribution of incentive salience to reward-associated stimuli (Robinson and Berridge, 2008; Fineberg et al., 2010; Pastor et al., 2010). Specifically, we used a drug-food cross-sensitization paradigm (cf. Nencini and Stewart, 1990; Bakshi and Kelley, 1994; Le Merrer and Stephens, 2006) to discriminate between excessive intake of pleasure-giving foods and addictive drugs. Our experiments demonstrated that mice of differing body mass (normal, overweight, obese) do not display food-morphine cross-sensitization. Since drug addiction may be considered to be an attempt to compensate for an underlying malfunction in reward pathways (Vanderschuren and Kalivas, 2000; Vezina, 2004; Pastor et al., 2010), we conducted a converse experiment to test whether morphine-sensitized mice would over-consume (substitute) a highly rewarding food (5% sucrose). Those experiments showed that morphine-sensitized and non-sensitized mice ingest similar amounts of sucrose under conditions of satiety and starvation and, further, that body mass does not influence reward consumption. Thus, i) unlike drugs of abuse, food does not induce behavioural sensitization, and ii) sensitization to a drug of abuse does not alter consumption of food in overweight mice. It thus appears that although the rewarding and motivational effects of food and drugs of abuse are mediated by similar neurochemical mechanisms, obesity and

drug addiction represent a summation of other dysfunctional input and output pathways that lead to the emergence of two distinct disorders.

It is prudent to note that, our cross-sensitization studies were done with the opiate morphine, a prototypic drug of abuse; this contrasts with the common use of cocaine (popularly used in drug abuse research) to investigate the question of whether feeding can become addictive. Like cocaine, morphine induces behavioural sensitization, cross-sensitization to other abused drugs and conditioned place preference and, is self-administered by experimental animals (Nencini and Stewart, 1990; Bakshi and Kelley, 1994; Le Merrer and Stephens, 2006). Monoaminergic systems are activated by morphine and cocaine, both of whose effects ultimately converge to increase dopaminergic signalling in the nucleus accumbens (NAc), albeit through different mechanisms: morphine disinhibits dopaminergic neurons in the ventral tegmental area by inhibiting γ-aminobutyric acid (GABA) interneuron activity, whereas cocaine increases dopamine at NAc terminals by inhibiting monoamine uptake (Koo et al., 2012). In light of these profiles, there is no *a priori* reason to expect that food-morphine and food-cocaine cross-sensitization should produce qualitatively different sensitization of the dopaminergic system, among others. Moreover, the suitability of using morphine in food-drug of abuse cross-sensitization studies has been demonstrated previously Nencini and Stewart, 1990; Bakshi and Kelley, 1994; Le Merrer and Stephens, 2006).

Observations that similar corticolimbic pathways are activated in obese subjects and subjects who are addicted to drugs of abuse (Volkow et al., 2013) have propagated the idea that addiction to energy-dense/sweet foods underlies human obesity. However, there is growing consensus that this parallel is misleading, aptly embodied in the statement that "food addiction is neither enough nor necessary to develop obesity in humans" (Epstein and Shaham, 2010). Results from studies in animals have also contributed to the "addiction hypothesis of obesity". However, critical appraisal of one of the key studies suggesting that rats can become addicted to sugar (Avena et al., 2008, 2012) has been challenged on the grounds that the animals in those studies did not gain body mass, most probably because they consumed less of their standard diet, the availability of which was restricted (Peters, 2011b; Ziauddeen et al., 2012; Ziauddeen and Fletcher, 2013). Another study reported compulsive eating and weight gain in rats on a cafeteria diet in which animals could choose between standard chow and a high-fat/high-sugar diet (Johnson and Kenny, 2010), but its conclusions do not distinguish between compulsive binge-eating and overeating on the one hand, and on the other, overeating which leads to obesity (Ziauddeen et al., 2012). Different physiological and neurobiological mechanisms are

likely to underlie the two disturbed patterns of eating and notably, only binge eating shares (some) characteristics with addictive processes in humans (de Jong et al., 2012).

In summary, we demonstrate that the display of ST behaviour in pavlovian conditioning does not indicate susceptibility to overeat. This, together with our observation that cross-sensitization between food and drugs of abuse does not occur, adds important new evidence to the debate about whether eating can become an addictive behaviour and lead to obesity (de Jong et al., 2012; Ziauddeen and Fletcher, 2013). Based on this, we suggest that pharmacological treatments designed for drug abuse are unlikely to be effective at reducing overeating and thus, overweight and obesity. Our results may help change patients' and Society's perception of overeating as a distinct disorder that does not carry the stigma still attached to addictive disorders. Nevertheless one caveat with respect to our work is that results from experiments in laboratory animals cannot be directly extrapolated to understanding human obesity: while the former eat what is provided in order to survive, they do not experience the natural hazards faced by free-foragers and lack the abundance and choice of foods enjoyed by humans living in industrialized societies.

CHAPTER 3

Altered motivation, matching energetic need rather than hedonic value, masks appetitive learning potential of obese mice

Adapted from:

Mazen R. Harb and Osborne F. X. Almeida (2014) Altered motivation, matching energetic need rather than hedonic value, masks appetitive learning potential of obese mice. *Frontiers in Behavioural Neuroscience* (Manuscript under review).

3.1 ABSTRACT

Hunger motivates feeding behaviour, which itself represents an integration of physiological and neural signals. In humans, conditioning by environmental cues can trigger "hedonic overdrive" and thus, excessive eating and weight gain. Mice are frequently used to explore the regulation of human appetite but it is not known whether their conditioned learning of, and motivation for, food rewards varies as a function of body mass. To address this, we tested adult male mice of different body weights (by maintenance on diets of differing caloric contents) in two appetitive conditioning paradigms (pavlovian and operant) and, in food retrieval and hedonic preference tests, in an attempt to dissect the respective roles of learning and motivation and energy state in the regulation of feeding behaviour. We show that 1) the rate of pavlovian conditioning to an appetitive reward develops as an inverse function of body weight and, mice with higher body weights display a greater latency to collect food reward; 2) overall, mice with lower body weights show greater motivation to work for a food reward (faster acquisition of the operant conditioning procedure, lower latencies to approach and retrieve the reward and more food-tray entries), as compared to animals with higher body weights and, 3) in a preference test setting, overweight and obese mice show lower consumption of palatable foods (isocaloric milk or sucrose) compared to controls in the presence or absence of maintenance chow; however, all groups of mice adjust their consumption of the individual food types, such that their total daily caloric intake relative to body weight remains constant, irrespective of weight status. Thus, in contrast to humans, mice regulate their caloric intake according to their metabolic status rather than to the hedonic properties of a particular food. This ability may mask the fact that overweight and obese mice do not have a reward deficiency syndrome or reduced capacity for appetitive learning.

3.2 INTRODUCTION

Ingestion of foods in excess of actual energy needs leads to overweight and obesity, conditions that raise an individual's risk to develop non-communicable chronic physical and mental diseases (Danaei et al., 2011; Gunstad et al., 2010; Moussavi et al., 2007; Wormser et al., 2011). To help stem the worldwide rise in overweight and obesity (WHO, 2000), it is imperative to further our understanding of eating behaviour. Feeding is an innate behaviour, involving cognitive (attention, learning and memory, decision-making), sensory (olfactory, visual, taste, somatosensory) and behavioural (motivation) processes that work in an inter-dependent manner (e.g. motivation can be elicited by novel or previously-learnt sensory rewards) (Berthoud, 2011). Importantly, feeding behaviour is also governed by peripheral signaling to the brain about energy levels and satiety state (Berthoud, 2011). Thus, the amount of food consumed by an individual is determined by convergence and integration of a multiplicity of neural and peripheral signals and execution commands that are not easy to dissect.

"Hedonic overdrive" has been recently proposed as an explanation for overeating in humans (Berridge and Kringelbach, 2011; Cohen, 2008; van der Plasse, 2013). Briefly, the high reward salience of certain foods leads to their consumption even in states of satiety and/or sufficient energy reserves. Responses to hedonic stimuli depend largely on conditioned learning of environmental cues, well-exemplified by the impact of advertising on food choices and intake (Boyland et al., 2011; Halford et al., 2008; Jones et al., 2010; Petrovich et al., 2007b; Powell et al., 2010). One important question in the field relates to the mechanisms that drive excessive eating in overweight and obese human subjects, i.e. Why can overweight and obese individuals not exert sufficient control over their responses to pleasurable foods? Since excess body weight can reportedly interfere with cognitive performance, is it plausible, for example, that overweight subjects continue to be more susceptible to conditioning stimuli? (Cohen, 2008; Jansen et al., 2003; Rothemund et al., 2007). Another possibility is that overeating in a state of satiation or in the presence of sufficient energy depots is a sign of "reward deficiency syndrome" and reflects dysregulated motivation (Blum et al., 2006; Geiger et al., 2009; Stice et al., 2008; Wang et al., 2004). These possibilities are by no means exhaustive and may include other deficits, including disrupted energy mobilization and energy sensing.

Laboratory rodents are frequently used in research aimed at dissecting the neural and physiological mechanisms that control feeding behaviour and body weight (Speakman et al., 2007). Many published

studies have demonstrated that obesity compromises memory (Farr et al., 2008; Greenwood and Winocur, 1990; McNeilly et al., 2011; Mielke et al., 2006; Murray et al., 2009; Valladolid-Acebes et al., 2011). Usually, however, the tests use food as the reinforcing stimulus and interpretation of the results do not consider that obese animals have abundant energy supplies and may therefore be less motivated to perform (Kubera et al., 2012; Peters et al., 2004; Peters and Langemann, 2009; Shin et al., 2011). As a result, the idea that obese animals do not perform well because their cognition or reward sensitivity is disturbed may be misleading and, when translated to humans, may stigmatize persons with eating disorders (Puhl and Heuer, 2010).

The experiments reported here were aimed at clarifying the relative roles of cognition, motivation and energetic state in the control of feeding behaviour in adult mice that were of normal body weight, overweight and obese; the last two groups of animals were generated by exposing them to energy-rich diets. Our results show that motivation, and therefore learning in an appetitive conditioning task, is inversely proportional to body weight; notably, body weight generally correlates with total fat mass (see (Hariri and Thibault, 2010)), fat being a primary energy depot. Moreover, our results demonstrate that mice can trade off the hedonic properties of palatable foods (e.g. milk, sucrose) for energy-denser maintenance diets so as to meet their actual energy needs. In this respect, humans and mice may differ remarkably.

3.3 METHODS

Animals: Male mice (C57BL6 strain, Charles River, Sulzfeld, Germany) were used in these experiments. Animals were housed in pairs under standard laboratory conditions with ad libitum access to water, unless specifically mentioned. Experimental procedures were compliant with European Union Directive 2010/63/EU and local regulations.

Variable degrees of overweight were induced by maintaining mice on either a standard laboratory (normal) chow (NC; 11.9 kJ/g, 19% crude protein, 4% crude fat, 6% crude fiber), a low-fat, high-carbohydrate diet (LF-HC; 16.1 kJ/g, 10% from fat, 70% from carbohydrate), or a high fat-high, high-carbohydrate diet (HF-HC; 19.8 kJ/g, 45% from fat, 35% from carbohydrate). The NC was purchased from Altromin (Lage, Germany, diet 1324 TPF); LF-HC and HF-HC diets were supplied by Brogaarden (Lynge, Denmark, diets D12450B and D12451, respectively, from Charles River Laboratories). Animals received the diets for 12 (pavlovian and operant conditioning experiments and motivation/"wanting" tests) or 36 (hedonic preference/"liking" tests) weeks, from 3 months of age onwards.

Behavioural tests (open field, pavlovian and operant conditioning and tests of motivation and preference) were conducted during the daily phase of darkness (lights off: 07:00). Bussey-Saksida automated touchscreen chambers (Horner et al., 2013) were used for the pavlovian and operant conditioning experiments, as described previously (Harb and Almeida, 2014). The reward used as a reinforcer in pavlovian and operant conditioning and motivation test was a liquid food (15 µl of diluted condensed milk, containing 14% sugar). Before any testing commenced, animals underwent 1 week of habituation to the experimental room and experimenter as well as to the liquid food rewards in the test chambers. All animals were subjected to a calorie-restriction schedule to reduce body weights by 10-15% before behavioural testing and calorie restriction continued throughout, unless otherwise stated.

Open field test: The open field (OF) test was used to measure locomotor activity and explorative behaviour; this test was used to ensure that the different diets and induced changes in body weight did not interfere with the animals' motor abilities or, indirectly, with their attention and motivation states. The OF test was conducted before all other behavioural tests. Testing was done in a white light-illuminated (100 lux) Plexiglas arena (OF; white base: 30 X 30 cm; dark grey walls: 30 cm high), in an otherwise dark room. Activity was recorded over 5 min. using a video camera and results were analyzed using ANY-maze software (Stoelting, Wood Dale, IL). The total distance travelled by each

mouse was computed. Mice were placed in the OF arena (5 min/session/d) on 2 consecutive days; the first session was used to habituate the animals to the test environment.

Pavlovian conditioning: The autoshaping paradigm followed has been described previously (Harb and Almeida, 2014). Briefly, a 10 s flash of white light, in either the left- (50% of animals) or right- (50% of animals) hand side of the screen, was used as the conditioned stimulus (CS); immediately following the light flash, a liquid food reward (unconditioned stimulus; US) was delivered into the food magazine. Mice were exposed to these parameters once daily during which time they were presented with 15 CS+ (light stimulus followed by reward delivery) and 15 CS- (light stimulus without reward delivery) in a randomized order; (maximum of 2 consecutive presentations of same stimulus, VI schedule of 10-40 s between each stimulus). Animals were considered to be Sign Trackers (ST) upon reaching the criterion of 70% correct responses/session to the CS+ on at least 3 consecutive days. Animals that made <20% approaches to the CS+ were categorized as Goal Trackers (GT), and those that made 20-65% approaches to the CS+ were considered to be Intermediate Trackers (IT) (see Harb and Almeida, 2014).

Operant (instrumental) conditioning: A separate batch of animals was used for this set of experiments. Daily sessions comprised of 20 presentations of a light stimulus at the center of the touchscreen. Animals had to "work" for a reward, delivered in a food-tray at the opposite end of the chamber by nose-poking the stimulus; reward delivery was made as soon as the stimulus was touched (Horner et al., 2013). In order to minimize between-trial interference, a variable interval (VI) schedule (10-40 s) was used. Each mouse experienced 1 daily conditioning session that lasted a maximum of 60 min until it reached criterion (completion of 20 trials in < 20 min/session on at least 3 consecutive days). The following parameters were recorded and computed for each operant conditioning session: (i) trials completed/ session, (ii) time to complete session, (iii) beam breaks/min, and (iv) stimulus touches/min.

Tests of motivation and hedonic preference: Motivation for food reward retrieval was examined in two ways:

(i) Motivation (Harb and Almeida, 2014) was evaluated by monitoring reward retrieval latencies and rate of food-tray entries in touchscreen chambers (Horner et al., 2013). Testing was carried out over 2

daily sessions, each of which consisted of 15 presentations of liquid food reward, delivered at a variable interval (VI) 10-40 s, independent of learning strategies, and only after retrieval of the previously-delivered reward.

(ii) Hedonic preference was examined in a batch of mice that had been maintained on NC, LF-LC or HF-HC diets for 36 weeks, starting at 3 months of age. Mice were presented with two highly-rewarding isocaloric drinking solutions (15% sucrose or milk whose fat content was 5%) in their home-cages; maintenance chow/water being available ad libitum throughout, and fluid consumption was measured at 3, 6 and 24 h. In brief, this protocol allowed assessment of the hedonic preference of the liquid diets, independently of the animals' state of satiety or energy needs. In a second step, mice were food-deprived for 48 h and allowed to choose between the milk and sucrose solutions; testing was done in the home-cage but animals did not have access to their normal chow. This design allowed discrimination between hedonic preference vs. energy needs by computing actual calories derived from (each of) the liquid foods as a function of the average daily number of calories derived from the maintenance (NC, LF-HC, HF-HC) chow under normal holding conditions.

Data analysis: Data analyzed using the statistical software package Prism 5.0 (GraphPad, La Jolla, CA). Data were initially subjected to ANOVA, followed by appropriate post-hoc comparisons. The minimum level of significance was set $p < 0.05$.

3.4 RESULTS

Inverse relationship between efficacy of conditioning to food cues and body mass

To address the hypothesis that appetitive learning is altered in overweight and obese individuals, we here applied the classical pavlovian conditioning paradigm to mice that differed in body mass, reflecting their maintenance on normal chow (NC) (CON, hereinafter referred to as "control mice", N =18), low-fat/high-carbohydrate (overweight, N = 16) or high-fat/high-carbohydrate (obese, N = 15) diets. Body weights differed significantly between each of the experimental groups ($P < 0.001$, Fig. 3.1A); none of the groups displayed motor or other behavioural impairments, as indicated by the results of testing in an open field arena (Fig. 3.1B).

Mice were trained over 11 days (15 CS+ and 15 CS- presentations/trial session). Consistent with our earlier findings (Harb and Almeida, 2014), all control mice showed conditional learning, albeit by developing three distinct patterns of conditioned responses (CR; sign tracking [ST], goal tracking [GT] and intermediate tracking [IT], showing 65%, < 20% and 20-65%, respectively, of approaches to the CS+) (Fig. 3.1C). In contrast, 94 and 70% of the overweight and obese animals, respectively, displayed conditioned behaviours, with 70% of each group showing IT-type of CR (data not shown).

Given the above observations and to examine whether the different CR patterns of the three groups reflected reactivity to the test set-up, rather than differences in learning *per se*, we compared session completion times and latencies to reward collection. Control, overweight and obese animals differed significantly in the time taken to complete the training sessions ($F_{2,429} = 119.3$; $P < 0.0001$; Fig. 3.1D). During all sessions, obese mice took significantly longer to complete the training session, as compared to control mice (session 1: $P < 0.05$; sessions 2-4, 9-11: $P < 0.001$; sessions 5,6,8: $P < 0.01$). Generally, the overweight animals were also slower than control mice in session completion (session 2: $P < 0.01$; sessions 3,4: $P < 0.001$), and significantly faster than the obese group on the last day of training (session 11: $P < 0.001$). Between-group differences were also detected in terms of another test parameter, namely, latency to collect reward ($F_{2,409} = 52.8$; $P < 0.0001$; Fig. 3.1E). *Post hoc* analysis revealed shorter latencies in control *vs.* overweight mice during sessions 1 ($P < 0.001$), 3 ($P < 0.05$) and 4 ($P < 0.001$); reward collection also occurred faster in control *vs.* obese mice during sessions 1 ($P < 0.001$), and 8 and 11 ($P < 0.001$).

Together, the above sets of data show weaker acquisition of an appetitive learning task by overweight and obese mice, possibly due to overall reduced reactivity to the task.

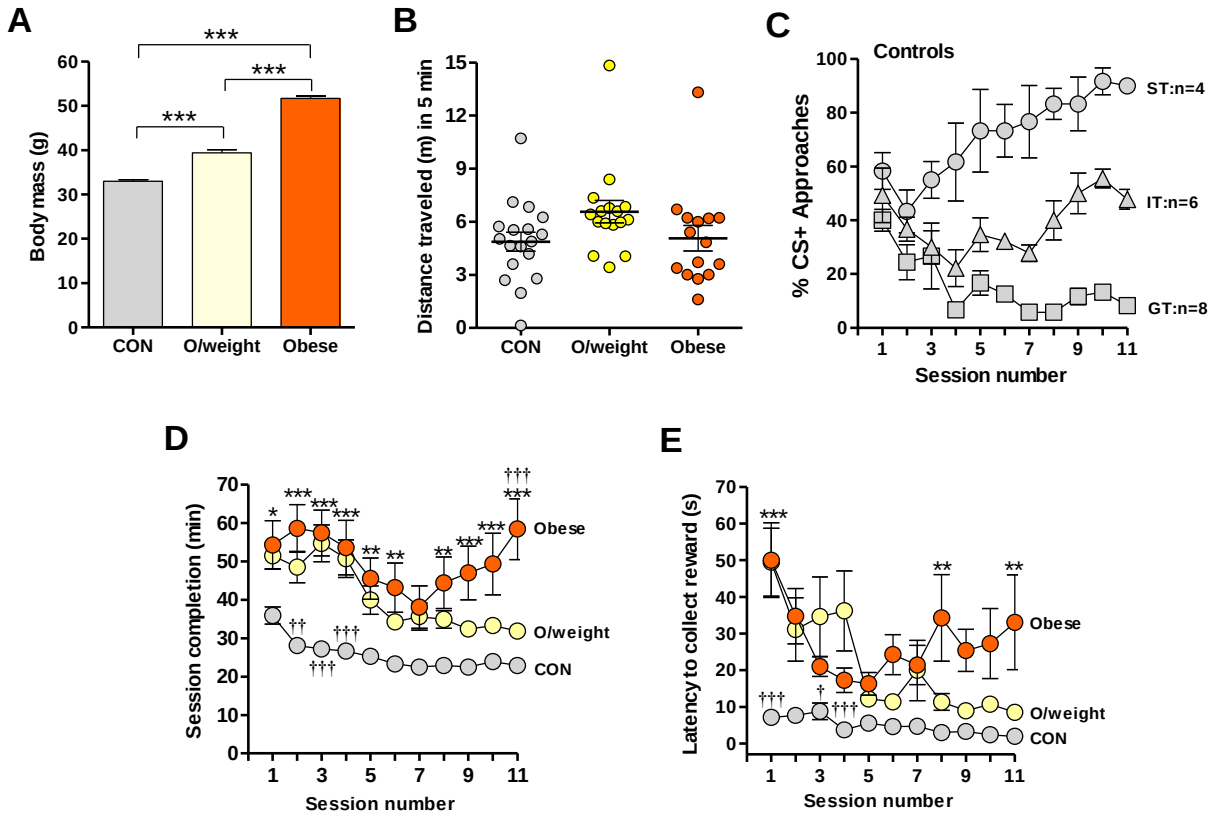

Figure 3.1 Overweight and obese mice show poor acquisition of food-rewarded pavlovian conditioned learning. (A) Body masses of control (CON; normal chow, n = 18), overweight (O/weight; low fat-high carbohydrate diet; n = 16) and obese mice (high fat-high carbohydrate diet, n = 15) at the start of experimentation. **(B)** Locomotor activity, measured in an open field arena, of CON, O/weight and Obese mice before behavioural testing commenced. **(C)** Relative number of CS+ approaches during each session; only CON mice displayed different conditioned responses (cf. Harb & Almeida, 2014), characterized as sign-tracking (ST, predominantly approached the CS+; n = 4), goal-tracking (GT, predominantly approached the US; n = 8), and intermediate-tracking (IT, alternated between CS+ and US with approximately equal frequency; n = 6). Autoshaping was monitored over 11 sessions; in each session, mice received 15 CS+ and 15 CS- presentations. **(D)** Time in min needed to complete successive autoshaping sessions. **(E)** Mean latency (s) to retrieve food reward during consecutive training sessions. Data are means ± SEM. *** in **A** denotes $p < 0.001$. *, ** and *** in **(D, E)** indicate differences between CON and obese groups at $p < 0.05$, 0.01 and 0.001, respectively. †, †† and ††† in **(D, E)** indicate differences between O/weight mice *vs.* CON and Obese mice at $p < 0.05$, 0.01 and 0.001, respectively.

Operant conditioning performance declines with increasing body mass

In an attempt to better understand the results obtained in the pavlovian conditioning experiments, we tested the performance of control, overweight and obese mice in an operant (instrumental) conditioning paradigm. Operant conditioning is another form of associative learning that requires subjects to perform an action in response to a stimulus (here, nose-poking the illuminated area of a touchscreen) in order to gain a reward (sweetened milk). Testing was done over 9 consecutive daily sessions, each comprised of 20 trials (20 presentations of the light stimulus). The criterion was that all 20 trials in a session should have been completed within 20 min on 3 consecutive days.

Most (87.5 %, 14/16 mice) control mice reached criterion, i.e. were efficiently conditioned. However, only 43 % (7/16 mice) of the overweight mice and none (0%, 0/16 mice) of the obese mice were conditioned. Session completion rates differed significantly between animals of different body mass in the following (increasing) rank order: controls (*vs.* overweight mice in sessions 3,4: $P < 0.001$; session 5: $P < 0.01$; session 6: $P < 0.05$; and *vs.* obese mice in all sessions: $P < 0.001$), overweight (*vs.* obese mice in sessions 5,7,8: $P < 0.05$) and obese (Fig. 3.2B). Notably, the obese group was slower in acquiring the task, with none of the animals in this group being able to complete all 20 trails/session during the first 4 days of testing (Fig. 3.2A). On the other hand, despite overall (all sessions) significant between-group differences ($F_{2,405} = 150.9$; $P \leq 0.0001$), all groups took progressively less time to complete the task ($F_{8,405} = 7.4$; $P \leq 0.0001$) (Fig. 3.2B).

To exclude impairments in motor activity and/or lack of interest that could potentially account for the slower learning by overweight and obese mice, we monitored the rate of photobeam breaks and stimulus touches (nose-pokes). While the overweight and obese mice were less mobile (fewer photobeam breaks), as compared to controls, during the first three test sessions, none of the groups differed in locomotor activity between sessions 4 and 9 (Fig. 3.2C). Although ANOVA revealed a significant overall increase in stimulus nose-poking over time ($F_{8,405} = 9.1$; $P \leq 0.0001$); Fig. 3.2D shows that this increase was mainly attributable to the control and overweight groups ($F_{2,405} = 77.8$; $P \leq 0.0001$), the obese animals showing significantly fewer nose-pokes than controls during sessions 3-9 ($P < 0.001$) and overweight mice during sessions 4,5 and 8 ($P < 0.05$).

The results from these experiments indicate that higher body mass is associated with lower interest in an operant task in which food is provided as the reward; our results rule out impaired motor ability in overweight and obese mice, but do not exclude the possibility that they suffer from a deficit in motivation to work for food.

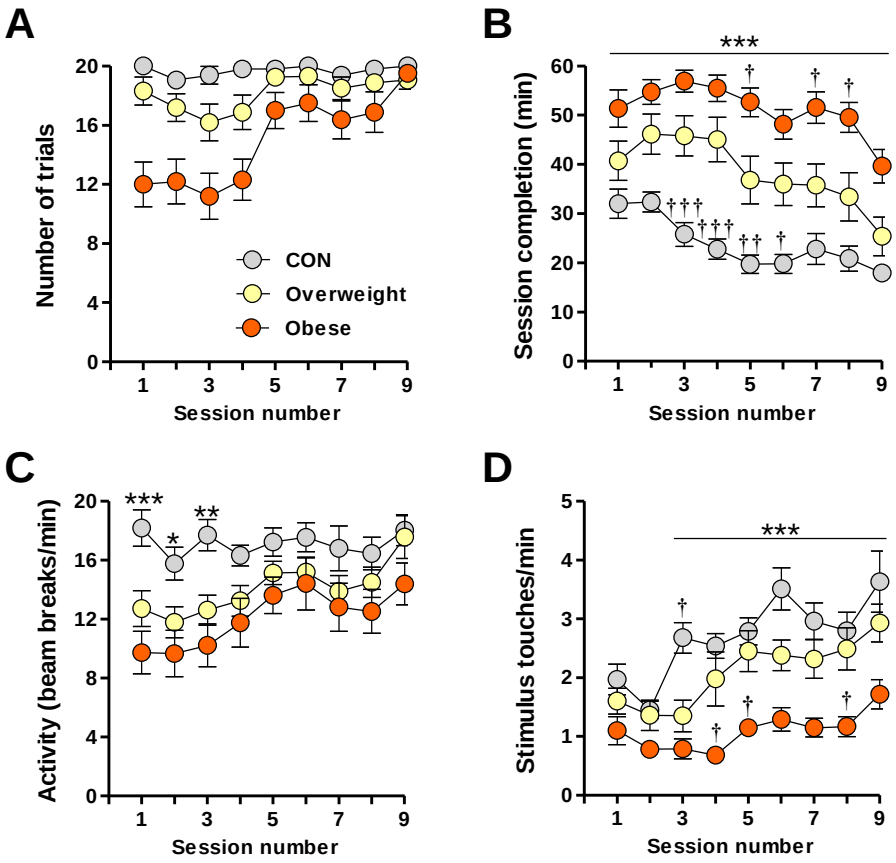

Figure 3.2 Impaired learning of a food-rewarded operant conditioning task by overweight and obese mice. Control (CON), overweight (O/weight) and obese mice (n = 16/group) were tested in 9 consecutive sessions, each consisting of 20 trials. Data shown are **(A)** Number of trials completed per test session; **(B)** Time (min) required to complete each successive session; **(C)** Locomotor activity (infra-red beam breaks/min) in touchscreen test chamber; **(D)** Number of stimulus touches/min. Data is presented as means ± SEM. *, ** and *** indicate significant differences between CON and obese groups ($p < 0.05$, 0.01 and 0.001, respectively). †, †† and ††† denote significant differences between overweight mice *vs.* CON and obese mice ($p < 0.05$, 0.01 and 0.001, respectively).

Altered motivation for food in overweight and obese mice

Here, we specifically asked whether overweight and obese animals responded differently to controls in the food conditioning experiments because of a lack of motivation towards food stimuli, i.e. if their slower acquisition of the pavlovian conditioning paradigm, in which food served as the reward, was due to their reduced interest in food *per se*. Food-restricted animals were tested on two consecutive days (1 session/day). We assessed three parameters (latencies to approach the reward and retrieve it, and number of food-tray entries) that inform on motivation for food reward (sweetened milk, 15 deliveries/session).

Body mass had a significant impact on approach latency ($F_{2,71}$ = 137.5; $P \leq 0.0001$; Fig. 3.3A) and the time elapsed before reward retrieval ($F_{2,74}$ = 63.1; $P \leq 0.0001$; Fig. 3.3B). The obese mice approached the reward with a significant delay, as compared to the overweight ($P < 0.001$) and control ($P < 0.001$) mice; overweight mice also showed a higher approach latency than controls ($P < 0.05$) (Fig. 3.3A). Likewise, the rate of food reward retrieval and consumption was highest in controls > overweight > obese mice (Fig. 3.3B). Monitoring the rate of food-tray entries as an additional index of motivation for food, revealed significant between-group differences ($F_{2,72}$ = 25.3; $P \leq 0.0001$); the highest rate was seen in controls > overweight > obese mice (Fig. 3.3C).

The above findings suggest that lower motivation for an appetitive reinforcer, rather than impaired learning ability, can account for the poorer performance of overweight and obese mice in the pavlovian and operant learning tasks.

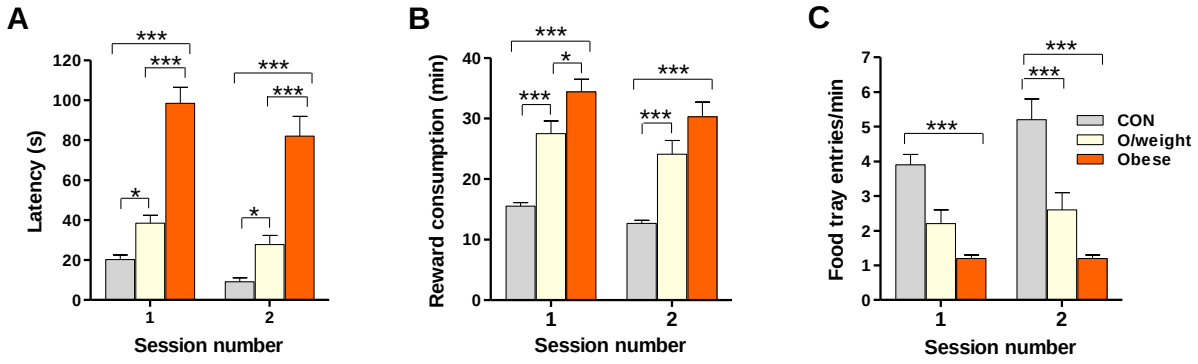

Figure 3.3 Overweight and obese mice are less motivated to collect palatable, but low-energy, food rewards. The sweetened milk reward was delivered 15 times in each session. The mean latencies to approach the reward **(A)**, times taken to retrieve (and consume) the reward **(B)**, and number of food tray entries **(C)** by CON (n = 18), overweight (O/weight, n = 14) and obese (n = 9) mice are shown (error bars represent SEM). * and *** represent significant differences between indicated groups at $p < 0.05$ and 0.001, respectively.

Mice adjust their consumption of palatable foods according to body mass

This experiment sought to examine whether the lower motivation seen in overweight and obese mice is related to their hedonic preference for palatable foods or to their higher body mass which, in turn, implies their higher energy depots (Hariri and Thibault, 2010).

In a first step, we monitored the 24 h consumption of two isocaloric liquid foods (15% sucrose and milk with a 5% content of fat) by 12-month old control, overweight and obese mice that had *ad lib* access to the experimental (NC, HF-HC or LF-HC) diets on which they had been maintained for 36 weeks. The three experimental groups differed in body weight (control: 42.8 $\pm$ 1.2 g; overweight: 49.9 $\pm$ 0.6 g; obese: 59.4 $\pm$ 0.8 g Fig. 3.4A). The groups also differed in their average daily intake of calories (relative to body weight, monitored over 3 consecutive days) and but the controls ingested significantly more calories than the overweight and obese groups ($P < 0.01$; Fig. 3.4B).

The temporal patterns of consumption of sucrose and milk by control, overweight and obese mice are depicted in Figs. 3.4C and 3.4D. Overall, the data show that, in contrast to humans/primates and rats (Levine et al., 2003; Naleid et al., 2008), mice prefer milk over sucrose. Nevertheless, all treatment groups consumed the sucrose solution (Fig. 3.4C), with control mice ingesting significantly more than the overweight and obese groups between 6 and 24 h [$F_{2,132} = 24.7$; $P \leq 0.0001$]; interestingly, the overweight mice ingested significantly more sucrose than their obese counterparts ($P < 0.001$). Obese mice consumed the least amount of milk, as compared to the control and overweight groups (6-24 h: $F_{2,132} = 15.3$; $P \leq 0.0001$; obese *vs* control.: $P < 0.001$; obese *vs.* overweight: $P < 0.001$) (Fig. 3.4D).

Expression of the total calorie intake, derived from the two liquid diets (sucrose and milk) and solid chow (NC, LF-HC, HF-HC), as a ratio of body weight (calories/g BW) revealed that control, overweight and obese mice consumed a similar relative number of calories during the 24 h test period (*inset,* fig. 3.4E). As shown in Fig. 3.4E, all animals derived the majority of their daily calories from their respective solid diets >> milk > sucrose ($P < 0.001$). Notably, the relative intake of calories from solid diet was significantly higher in the obese (HF-HC) *vs.* control (NC) and overweight (LF-HC) ($P < 0.05$) mice, and the relative intake of calories from sucrose was significantly lower in obese *vs.* control ($P < 0.01$) and overweight ($P < 0.05$) mice; these findings indicates that obese mice prefer the HF-HC diet over the hedonically-loaded foods.

The results of the above experiments suggested that food consumption in mice is based on the likelihood that the energy density of a particular food will fulfill its energy needs, rather than the sensory rewarding properties of that food. To explore this idea, we repeated the above food preference paradigm in control, overweight and obese mice that were previously food- deprived for 48 h (and did not have access to their respective solid diets during testing). This pretreatment was chosen to increase the motivation to eat as well as induce a relative energy deficit in all animals. As predicted, food deprivation caused a loss of body weight in all groups, the largest losses being observed in control and overweight mice ($P < 0.01$ *vs.* obese mice; Fig. 3.5A). Again, all treatment groups consumed fewer calories from sucrose (Fig. 3.5B) than milk (Fig. 3.5C), confirming their preference for milk (the liquid diets were isocaloric; preference in controls > overweight > obese mice; $F_{2,132} = 25.2$; $P < 0.0001$). The identical preference for milk over sucrose, by control, overweight and obese mice (Fig. 3.5D) demonstrates that the latter two groups do not have a reward deficit. Lastly, all groups of animals consumed a similar number of calories on 3 consecutive days at the end of the test phase of the experiment, at which time they were returned to their respective solid diets (controls: normal chow; overweight: LF-HC; obese: HF-HC), as shown in Fig. 3.5E.

In summary, the above findings show that increased body weight is not accompanied by a deficit in reward-responding (cf. Fig. 3.4 and Fig. 3.5) , and that mice have the capacity to regulate their food choices in a manner that maintains constant caloric intake relative to body mass (Fig. 3.4). The last point is reinforced by analysis and scrutiny of the data obtained in the tests of preference under conditions of *ad lib* access to one of three diets (NC, LF-HC and HF-HC, cf. Fig. 3.4) or under when animals were deprived of any solid food for 48 h (cf. Fig. 3.5).

Lastly, we calculated the relative amount of energy intake derived from each of the respective liquid and solid diets (controls: sucrose, milk and NC; overweight: sucrose, milk and LF-HC; obese: sucrose, milk and HF-HC) with respect to each group's average daily calorie intake (data in Fig. 4B). As shown in Fig. 3.6, control, overweight and obese mice can adjust the relative amounts of each liquid and solid diet in order to maintain a relatively similar daily level of calorie ingestion, irrespective of weight status. Together, these results show that animals with higher body mass do not have a reward deficit syndrome but neglect the otherwise highly-rewarding milk and sucrose in favor of their energy-denser solid foods.

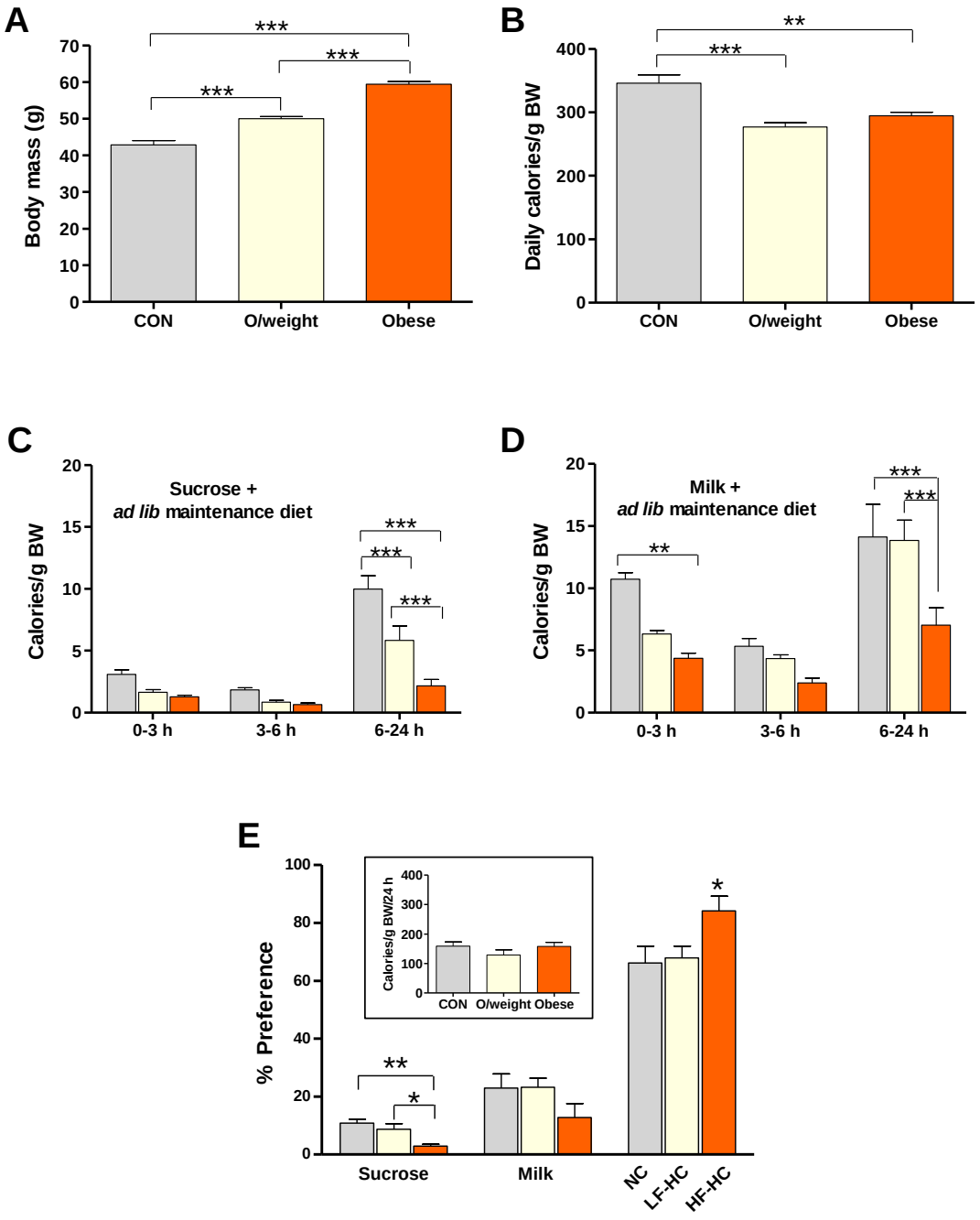

Figure 3.4 Mice of differing body masses are sensitive to the rewarding properties of both, low-calorie foods (isocaloric 15% sucrose solution and milk containing 5% fat) and energy-dense solid chow. Ingestion of the different foods was monitored in control (CON, n = 15), overweight (O/weight, n = 18) and obese (n = 14) mice during hours 0-3, 3-6 and 6-24 of presentation of the liquid foods *and* their maintenance solid diet (NC, LF-HC, HF-HC). **(A)** Body masses of the 3 groups of mice at the start of the experiment. **(B)** Average daily ingestion of calories from maintenance diets, corrected for body weight; data from 3 consecutive 24 h periods. **(C, D)** Body mass-corrected calories derived from sucrose or milk consumption over a 24 h period. **(E)** Preferences of CON, O/weight and obese mice for sucrose, milk and maintenance diet. The *inset* shows the total amount of energy ingested (maintenance diet + sucrose + milk) over 24 h. Depicted data are means ± SEM. *, ** and *** represent significant differences between indicated groups at $p < 0.05$, 0,01 and 0.001, respectively.

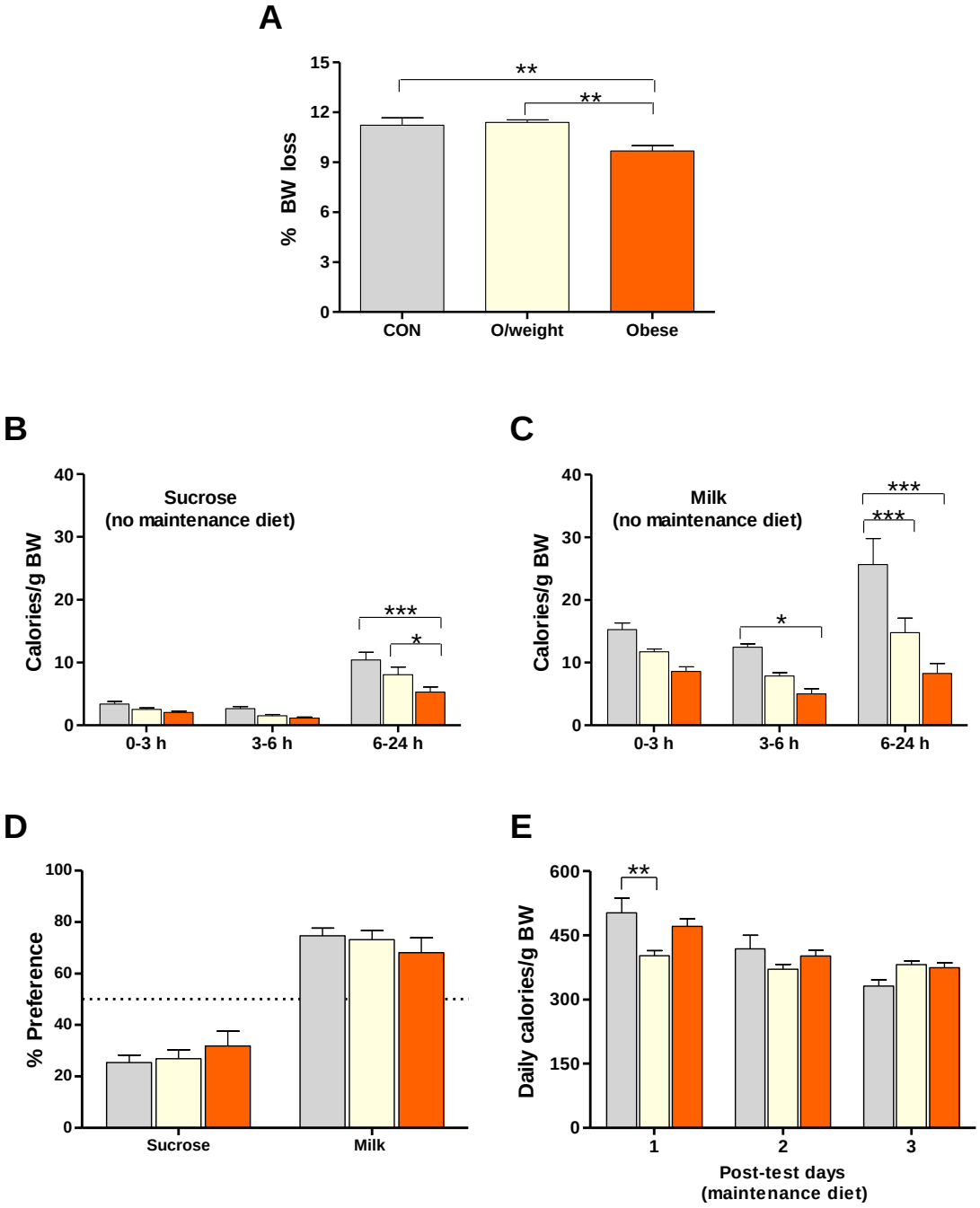

Figure 3.5 Overweight and obese mice display differential preferences for isocaloric foods that differ in their sensory (hedonic) properties. Isocaloric sucrose (15%) and milk (5% fat) were presented to control (CON, n = 15), overweight (O/weight, n = 18) and obese (n = 14) mice that had been deprived of their maintenance solid diet (NC, LF-HC, HF-HC) for 48 h. **(A)** Relative (%) body mass loss after 48 h food deprivation. **(B, C)** Calories derived from sucrose and milk over a period of 24 h. **(D).** Relative preference for sucrose and milk over 24 h ([calories derived from sucrose or calories derived from milk/total calories ingested] * 100). **(E).** Average number of calories derived from maintenance diet food on the 3 consecutive post-test days. Means ± SEM are shown. *, ** and *** denote significant (pair-wise) differences, where $p < 0.05$, 0.01 and 0.001, respectively.

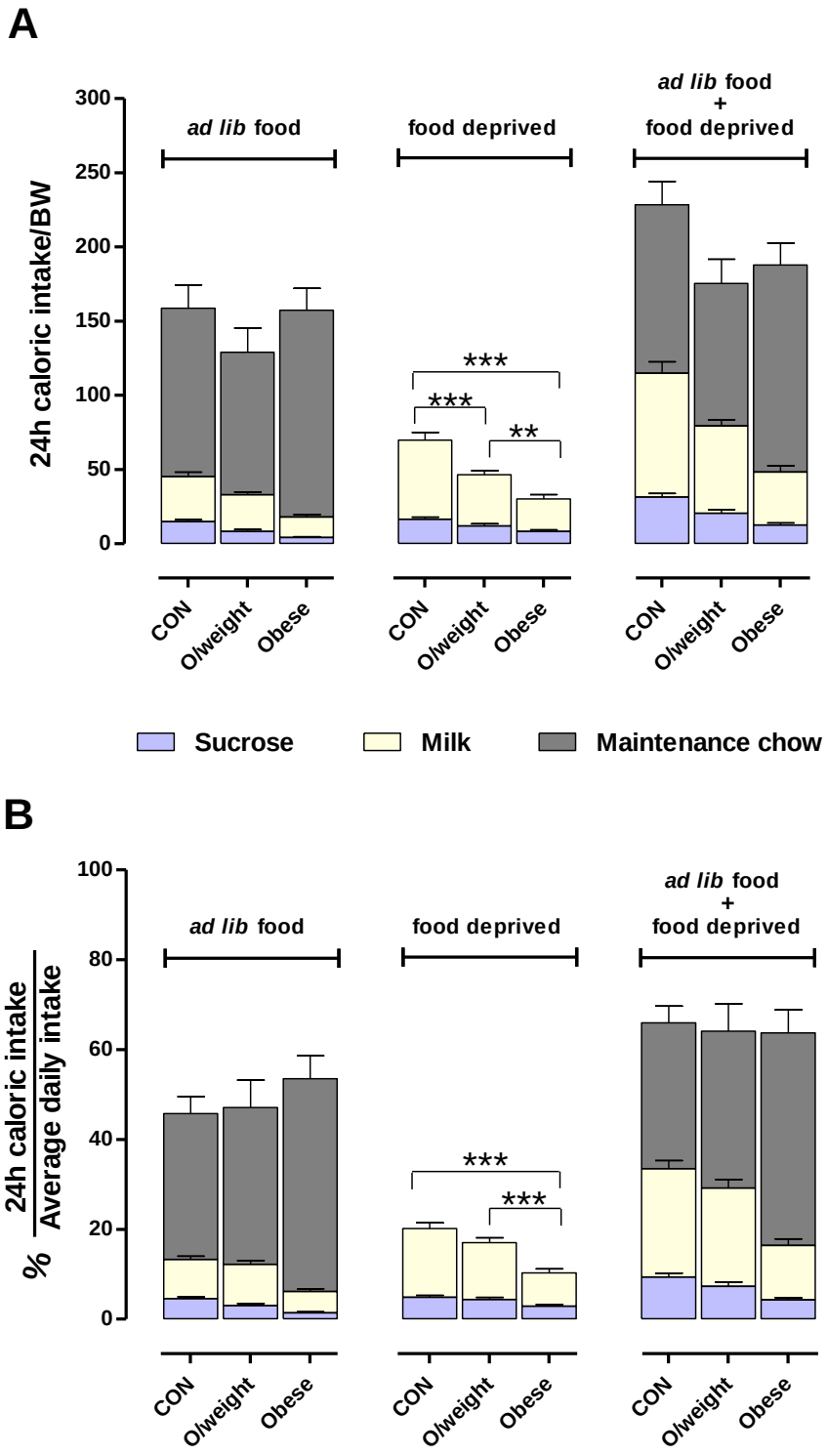

Figure 3.6 Mice adjust their intake of different foods to maintain a similar daily caloric intake relative to body mass. Comparisons between control (CON), overweight (O/weight) and obese mice are based on data depicted in Figs. 4 and 5. **(A)** Shows caloric intake during 24 h test phase from sucrose and milk, relative to body mass either in the presence of *ad lib* maintenance diet (NC, LF-HC, HF-HC) or in the absence of maintenance diet (food-deprived). **(B)** Shows caloric intake from sucrose and milk in *ad lib* presence or absence of maintenance diet, as a percentage of the average daily number calories consumed under standard feeding (solid chow only) conditions. Data shown are means ± SEM; significant pair-wise differences are denoted by ** ($p < 0.01$) and *** ($p < 0.001$).

3.5 Discussion

The behavioural mechanisms that lead to overeating and thus, overweight and obesity in humans, are still poorly understood. There are two prevailing hypotheses that are not necessarily mutually exclusive. The first posits that, overeating represents an addiction to food (compensation for an underlying reward deficit syndrome) (Blum et al., 2006; Geiger et al., 2009; Stice et al., 2008; Wang et al., 2004); the second suggests that physiological controls and signals of satiety are overridden by the hedonic (orosensory) properties of foods (Berridge and Kringelbach, 2011; Hariri and Thibault, 2010]. In a previous study in mice, a species increasingly used in research to understand human obesity (Speakman et al., 2007), we presented evidence that failed to support the addiction hypothesis of overeating (Harb and Almeida, 2014). The present study addresses two key facets of the hedonic overdrive hypothesis, namely, motivation and learning. These aspects are also pertinent since global cognitive (but not executive) function is reportedly disturbed in obese humans (Gunstad et al., 2010). However, a recent meta-analysis concluded that, whereas executive function may be compromised by obesity in children and adolescents, obesity does not have clear effects on other cognitive domains, such as learning and memory (Liang et al., 2014). Notably, increased body weight and sweet or fatty (high-calorie) diets have been shown to have a negative impact on the performance of laboratory animals in some (Beilharz et al., 2014; Farr et al., 2008; Heyward et al., 2012; Jurdak and Kanarek 2009; Ross et al., 2009; Stranahan et al., 2008; Valladolid-Acebes et al., 2013) but not all (Beilharz et al., 2014; Heyward et al., 2012; Hwang et al., 2010; Mielke et al., 2006; Ross et al., 2009; Valladolid-Acebes et al., 2013) tests of hippocampus-dependent spatial, recognition and fear learning and memory.

Our experiments show that overweight and obese mice perform poorly in pavlovian and operant conditioning, two paradigms that test appetitive learning paradigms. Complementary assessments of motivation revealed that this apparent impairment in learning ability results from the diminished motivation of overweight and obese animals to ingest food rewards. Importantly, we demonstrated that the reduced motivation to consume a food reward reflects reduced interest in appetitive reinforcers (sucrose and milk) that, although usually considered to be highly palatable and preferred (Lucas et al., 1998), contain less energy than maintenance (NC, LF-HC, HF-HC) chow in the amounts provided in the present experimental setting (cf. Fig. 3.5: reinforcers presented in the absence of maintenance diet; Fig. 3.4, reinforcers and maintenance diets available). A previous independent investigation, done in a

different context, in obese mice concluded that weight gain can occur despite reduced motivation to retrieve a hedonic food when the cost of acquiring energy-dense foods is low (Frazier et al., 2008). Together, these findings indicate that overweight and obese mice do not suffer from a reward deficit syndrome (cf. (Davis et al., 2008; Fulton et al., 2006; Geiger et al., 2009; Huang et al., 2005; Stice et al., 2008)); they can sense and respond to both, the sensory and energy signals elicited by foods, but are more likely to select foods that will match their metabolic status and fulfill their energetic demands.

The fact that overweight and obese animals worked less (i.e. were less motivated) for hedonically-loaded foods (sucrose, milk) may be explained by their greater energy depots stored in fat (Hariri and Thibault, 2010). This interpretation is supported by previously-observed lower motivation for an otherwise highly-palatable food in obese rats (Shin et al., 2011). Taken together, it thus appears that mice can adjust their food choices (in terms of hedonic and energetic properties) according to their actual energy needs. This point is illustrated by our observation that control, overweight and obese only differ in the amount of energy derived from individual foods, rather than in the total number of body weight-adjusted calories ingested (Fig. 3.6). It is important to note here that, laboratory animals may differ from humans in that they are less exposed to environments where hedonic signals abound and can override actual metabolic demands.

The present findings raise important questions regarding the interpretation of results from overweight and obese rodents in which learning and memory is assessed using paradigms in which food is used as the reinforcing stimulus (e.g. (Farr et al., 2008; Greenwood and Winocur 1990; McNeilly et al., 2001; Mielke et al., 2006; Murray et al., 2009; Valladolid-Acebes et al., 2011)). The apparent impaired ability of overweight and obese animals in such tests may simply reflect their reduced motivation (reduced "wanting") to retrieve and consume appetitive rewards, illustrated by our results from a motivation task that did not depend on learning ability (Fig. 3.3). This interpretation relates to behaviour in animals that are *already* obese or overweight, and not to the initiation of these states which result from multifactorial physiological and behavioural mechanisms (see (Hariri and Thibault, 2010)).

In summary, our experiments indicate that appetitive learning mechanisms are intact in overweight and obese animals, although over-shadowed by alterations in motivation (not reward insensitivity or reward deficit) for foods that may be hedonically less-attractive but more likely to meet the organism's metabolic needs. Unlike humans, mice eat according to their metabolic need rather than simply

respond to the hedonic properties of food. Our findings also show that extrapolation of results from studies reporting learning deficits in overweight/obese rodents to humans require caution; whereas most tests of learning ability in rodents employ appetitive stimuli, learning deficits in humans are detected using tests that are not confounded by the use of food-related stimuli. Lastly, translational studies need to recognize that humans are more exposed to reinforcing conditioning stimuli than laboratory animals and are therefore more likely to lose control over eating and gain excess weight.

CHAPTER 4

Reward components of feeding behaviour are preserved during mouse ageing

Adapted from:

Mazen R. Harb, Nuno Sousa, Joseph Zihl, Osborne F. X. Almeida (2014) Reward components of feeding behavior are preserved during mouse aging. *Frontiers in Aging Neuroscience*. (Manuscript under review).

4.1 ABSTRACT

Eating behaviour depends on associations between the sensory and energetic properties of foods. Healthful balance of these factors is a challenge for industrialized societies that have an abundance of food, food choices and food-related cues. We here asked, Does age influence appetitive conditioning? Operant and pavlovian conditioning experiments (rewarding stimulus was a palatable food) in male mice (aged 3, 6 and 15 months) showed that implicit (non-declarative) memory remains intact during ageing. Two other essential components of eating behaviour, motivation and hedonic preference for rewarding foods, were also found not to be altered in ageing mice. Specifically, hedonic responding by satiated mice to isocaloric foods of differing sensory properties (sucrose, milk) was similar in all age groups; importantly, however, this paradigm disclosed that older animals adjust their energy intake according to energetic need. Based on the assumption that the mechanisms that control feeding are conserved across species, it would appear that overeating and obesity in humans reflects a mismatch between ancient physiological mechanisms and today's cue-laden environment. The implication of the present results showing that ageing does not impair the ability to learn stimulus-food associations is that the risk of overeating in response to food cues is maintained through to old age.

4.2 INTRODUCTION

Metabolic status, a reflection of eating habits, is an important determinant of an individual's physical and mental health trajectory, especially from middle age onwards when the incidence of metabolic syndrome rises steeply (Mathus-Vliegen et al., 2012). Despite evidence linking overweight and obesity to increased risk for affective (Dixon et al., 2003; Preiss et al., 2013) and cognitive disorders (Dahl et al., 2013; Fitzpatrick et al., 2009; Smith et al., 2011; Ravona-Springer et al., 2013) and other age-related debilitating conditions (Gregor and Hotamisligil, 2011; Mathus-Vliegen et al., 2012), the neurobiology of eating behaviour as a function of age remains a relatively unexplored area; most published studies focus on understanding age-related loss of appetite and body weight (Frutos et al., 2012), rather than the rising tide of obesity in older individuals (Fakhouri et al., 2012).

Feeding is a motivated behaviour, driven by hunger (energy needs) but also by the reward salience of foods, represented by sensory (odor, visual appearance, taste and texture) and physical (energy content) attributes of a given food (Desmarchelier et al., 2013; Beeler et al., 2012; Rolls, 2010; Fernstrom et al., 2012; Mehiel and Bolles, 1988; Li et al., 2013). While hunger provides the primary motivation to eat, food-seeking (wanting/liking) and ingestive behaviour may be triggered by associations between the real or anticipated higher reward value of foods in a particular environmental context or because certain foods are imbued with hedonic properties (Berridge and Robinson, 2003; Berridge et al., 2009; Ferriday and Brunstrom, 2011; Ziauddeen et al., 2012). Conditioned (learned) stimuli can increase subliminal motivation to seek and consume foods during states of satiation and in excess of actual energetic demands, eventually leading to obesity.

Children and adolescents are more responsive and sensitive to external paired (conditioned) cues and the rewarding properties of palatable food than adults (Scully et al., 2012; Birch and Anzman-Frasca, 2011; Friemel et al., 2010). Normal ageing is accompanied by gradual structural and functional changes in brain areas involved in sensory, reward and cognitive processing (Marschner et al., 2005; Burke and Barnes, 2006). While age-related impairments of declarative and working memory have been extensively studied (Driscoll and Sutherland, 2005; Hedden and Gabrieli, 2004), little is known about the influence of ageing on non-declarative associative memory which is relevant to conditional learning (Birch and Anzman-Frasca, 2011; Petrovich, 2013). The present study examined whether implicit memory (associative learning), motivation and food preference (triggered by the food's

hedonic qualities) are affected during ageing in the mouse. Our results show that old mice do not suffer from impairments in their (i) ability to perceive hedonic stimuli, (ii) motivation to consume rewarding foods, and (iii) capacity to learn stimulus-food associations. Interestingly, mice also maintain their ability to adjust their calorific intake according to their metabolic status.

4.3 METHODS

Animals: Male mice (C57BL6 strain, Charles River, Sulzfeld, Germany), aged 3 (young), 6 (middle-aged) and 13-15 (old) months were used in these experiments. All procedures were carried out in compliance with national regulations on animal welfare and experimentation and European Union Directive 2010/63/EU. Animals were housed in pairs under standard laboratory conditions with ad libitum access to water, unless specifically mentioned. Behavioural tests (see below), each carried out in different batches of animals, were conducted during the animals' daily period of activity (diurnal phase of darkness; lights off: 7 a.m.) after 1 week of habituation to the experimental room and experimenter. In keeping with standard procedures, mice were placed on a calorie-restriction schedule to reduce body weights by 10-15% (body weights monitored daily), unless specifically stated otherwise. Animals that displayed overt signs of pathology (cf. Pettan-Brewer and Treuting, 2011) during autopsy at the end of each experiment were excluded from final analyses; the exact number of animals used in each experiment is given in the Results section and corresponding figure legends.

Operant (instrumental) conditioning: Tests were performed in automated touchscreen chambers (Horner et al 2013). The touchscreen, located opposite to the food magazine, was covered with a black Perspex mask, partitioned by three single rectangles. The unconditioned stimulus (white light) was presented through the middle partition only, a tone was presented when the mouse touched the screen with its snout. The stimulus was then extinguished and a liquid reward (15 µl of diluted condensed milk [14% sugar]) was delivered into the (now illuminated) food tray. A test session comprised 20 presentations of the light stimulus-reward delivery cycle. In order to minimize between-trial interference, a variable interval (VI) schedule (10-40 s) was used. Each mouse experienced 1 conditioning session/d that was terminated when criterion was reached (completion of 20 trials in < 20 min/session on at least 3 consecutive days) or after 60 min. Animals were habituated to the liquid reward and test chambers (3 d) before actual testing. The following parameters were recorded and computed during each operant conditioning session: (i) trials completed/session, (ii) time to complete session, (iii) beam breaks/min and, (iv) stimulus touches/min.

Pavlovian* (classical) *conditioning: Autoshaping was performed in automated touchscreen chambers, as previously described (Horner et al., 2013). The neutral stimulus (CS) was a 10 s flash of

white light in either the left- (50% of animals) or right- (50% of animals) hand side of the screen. Immediately after stimulus offset, a liquid food reward (15 µl of diluted condensed milk [14% sugar]) was delivered into the food magazine. During task acquisition mice were trained to associate the light stimulus (CS+) with reward delivery. During each session (1/day), presentations of 15 CS+ and 15 CS- were made in a randomized order (maximum of 2 consecutive presentations of same stimulus, VI schedule of 10-40 s between each stimulus). Animals reaching the criterion of 70% of correct [CS+] approach responses/session on at least 3 consecutive days were designated as sign trackers (ST) (Harb and Almeida, 2014).

Tests of motivation and hedonic preference: Two tests were used to examine motivation to retrieve a reward. The first was carried out over 2 d in the mouse touchscreen chambers; wanting/motivation was assessed by monitoring individual latencies to retrieve all of the reward and the rate of food tray entries. During each session, mice were presented with 15 liquid food rewards (15 µl condensed milk, containing 14% glucose), delivered at a variable interval (VI) 10-40 s. The second test was designed so as not to be confounded by satiety levels and energetic state. Specifically, it compared preference (liking) for one of two isocaloric drinking solutions (15% sucrose vs. milk whose fat content was 5%) presented in the home-cage. The test was conducted in a state of satiation and mice had access to their standard solid diets throughout the test. The volume of each liquid consumed was measured at 3, 6 and 24 h thereafter; the caloric intake provided by the liquid diets was computed as a function of standard chow consumption (weight and calories/body mass/d).

Data analysis: Data were subjected to statistical analysis using Prism 5.0 statistical software package (GraphPad, La Jolla, CA). Data were subjected to either 1- or 2-way ANOVA, followed by Bonferroni post-test comparisons, or by t-tests, as appropriate. The minimum level of significance was set to $p < 0.05$.

4.4 RESULTS

Operant conditioned learning is not impaired during ageing

Operant or instrumental conditioning involves learning to associate an action with an outcome; the paradigm requires that the subject "works" (here, nose-poking the light stimulus) in order to receive a reward (here, sweetened milk).

Calorie-restricted male mice aged 3, 6 and 15 months, hereinafter referred to as "young", "middle-aged" and "old" mice, were tested after 9 days when most (young: 100%, n = 18; middle-aged: 94%, n = 15; old: 92%, n = 12) had reached criterion (completion of 20 trials in < 20 min, on least 3 consecutive days) (Fig. 4.1A). Locomotor activity, measured in terms of photobeam breaks in the touchscreen chambers, did not differ between young, middle-aged and aged mice; all animals habituated to the experimental set-up similarly with a gradual, but significant, increase in locomotor activity over time [$F_{8,360}$ = 3.4; p = 0.0008 (Fig. 4.1B). Mice also displayed a progressive increase in the number of stimulus-directed nose-pokes over time [$F_{8,360}$ = 9.7; p < 0.0001] (Fig. 4.1C); however, the increase was more pronounced in the young and middle-aged mice [F2,360 = 24.2; p < 0.0001]. Overall, and irrespective of age, mice showed a progressive and highly significant decrease in the time needed to complete the daily sessions [$F_{8,361}$ = 40.1; p < 0.0001] (Fig. 4.1D). These results thus show that the capacity for operant learning does not deteriorate with ageing.

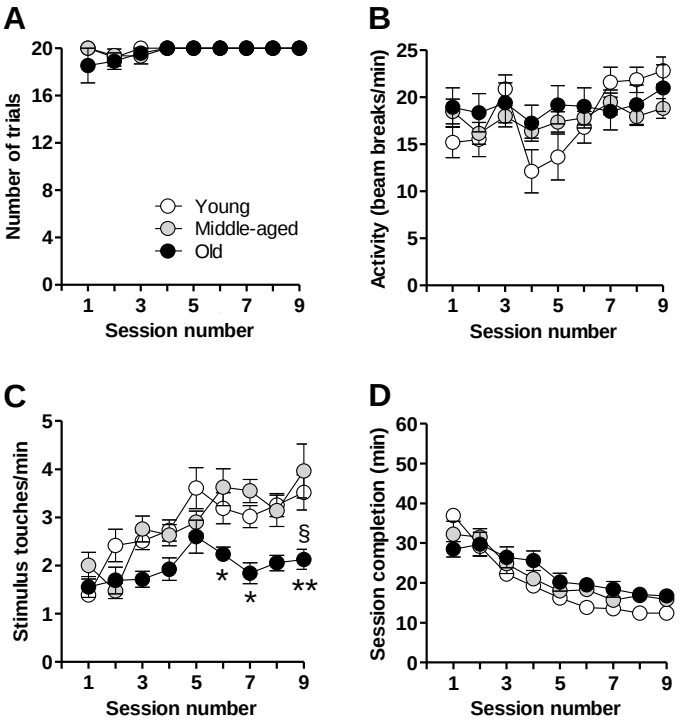

Figure 4.1 Acquisition of operant conditioning. Shown are the data in each of the 9 test sessions consisting of 20 trials in young (n=18), middle-aged (n=15) and old (n=12) mice. **A.** Number of trials completed. **B.** Locomotor activity (infra-red beam breaks/ min). **C.** Number of stimulus touches/ min. **D.** Time (min) for completion of session. Data are presented as means ± SEM. § indicates a significant difference (P < 0.05) between the young and old groups of mice. * and * * indicate significant differences (P < 0.05 and P < 0.01, respectively) between middle-aged and old mice.

Capacity for pavlovian conditioning does not change with ageing

Pavlovian conditioning represents another form of associative learning. It is highly conserved but individuals vary in stimulus-reward tracking patterns (Harb and Almeida, 2014). For the assessment of pavlovian conditioning, we compared the food cue-conditioned responses (CR) of young (n = 31), middle-aged (n = 34) and old (n = 34) male mice. In these experiments, a flash of light served as the neutral stimulus and liquid food (sweetened milk) was used as the rewarding stimulus.

Based on their CR on the last 3 days of the training schedule, mice were categorized as sign trackers (ST, at least 65% of approaches to CS+), goal trackers (GT, < 20% CS+ approaches), or intermediate trackers (IT, 20-65% CS+ approaches). Interestingly, segregation into ST, GT and IT was similarly distributed across all three age groups; young: 42% of mice were ST, 35% GT and 23% IT ($F_{2,303}$ = 409.8; $p \leq 0.0001$); middle-aged: 41% of mice were ST, 24% GT and 35% IT ($F_{2,316}$ = 78.9; $p \leq 0.0001$); old: 41% ST, 38% GT and 21% IT ($F_{2,328}$ = 133; $p \leq 0.0001$) (Fig. 4.2A-C), but the rate of task acquisition did not differ between age groups (Fig. 4.2D-F). Notably, while each of the three age groups had significantly different body masses, this parameter did not differ between mice displaying ST, GT or IT behaviour within each age group (data not shown). Briefly, the results of this test demonstrate that appetitive learning abilities are robustly conserved during ageing an d, that ST, GT and IT behaviours are innate characteristics that do not shift with age.

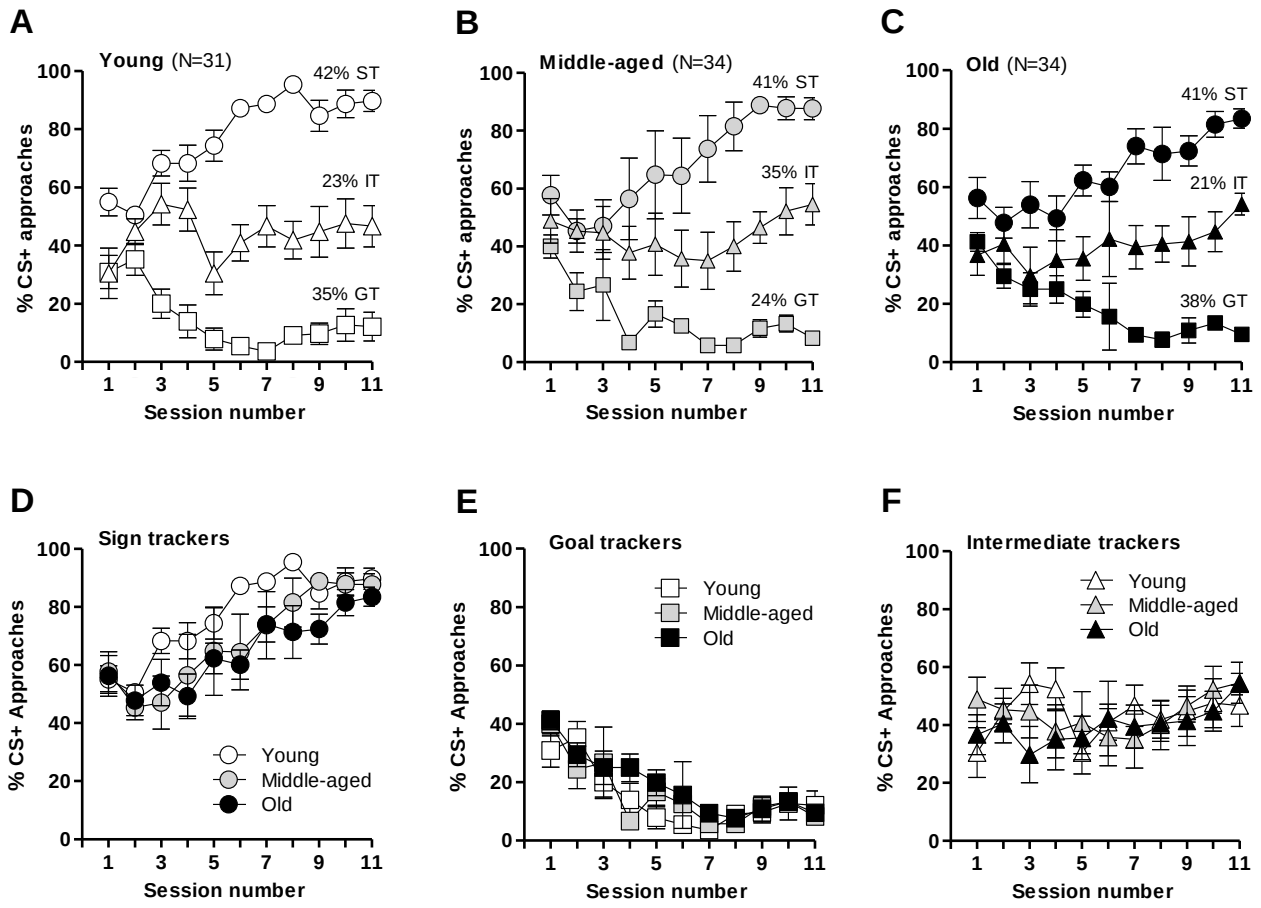

Figure 4.2 Acquisition of conditioned responses during autoshaping. Mice displayed different conditioned responses, designated as sign-tracking (ST, predominantly approached the CS+), goal-tracking (GT, predominantly approached the US) and intermediate-tracking (IT, alternated between CS+ and US with approximately equal frequency) behaviours. Autoshaping was monitored over 11 sessions; in each session, mice received 15 CS+ and 15 CS- presentations. **A.** The young mice (n=31) segregated into sign-trackers (ST; n = 13), goal-trackers (GT; n = 11) and intermediate trackers (IT; n = 7). **B.** The middle-aged mice (n=34) segregated into sign-trackers (ST; n = 14), goal-trackers (GT; n = 8) and intermediate trackers (IT; n = 12). **C.** The old mice (n=34) segregated into sign-trackers (ST; n = 14), goal-trackers (GT; n = 13) and intermediate trackers (IT; n = 7). **D-F.** CS+ approaches by, respectively, sign-, goal- and intermediate-tracking mice of different ages. Data are presented as means ± SEM.

Motivation for appetitive reward is intact in ageing mice

Motivation is a key factor in reward learning (Dayan and Balleine, 2002) and eating (Kringelbach et al., 2012) behaviour. Although the previous set of data showed that the capacity for implicit learning remains intact in aged mice, we consider of interest to examine whether ageing alters motivation for rewarding foods. To this end we monitored latency to approach, retrieve and consume sweetened milk (reward) and the number of food-tray entries in a food retrieval test, independent of learning strategies. The test was performed during a period of caloric restriction in young (n = 15), middle-aged (n = 16) and old (n = 15) mice; between-group starting body masses were significantly different (Fig. 4.3A; $p < 0.001$).

As shown in Fig. 4.3B, there were no significant age-related differences in latency of first approach-to-reward. Notably, however, approach latency decreased significantly, and in an "age-independent manner", in the second test session, indicating familiarity with the task and that it had been learnt ($F_{1,83} = 23.8$; $p \leq 0.0001$). Also, all age groups retrieved and consumed the sweetened milk reward within comparable times, their performance being significantly enhanced in the second test session ($F_{1,83} = 19.3$; $p \leq 0.0001$) (Fig. 4.3C). Significant between-age group differences were observed in the number of food-tray entries ($F_{2,83} = 19.7$; $p \leq 0.0001$) (Fig. 4.3D): young mice entered the food-tray more frequently than old mice on both test days ($p < 0.001$) and more frequently than the middle-aged group on the first day of testing ($p < 0.05$), consistent with the greater exploratory activity generally observed when younger mice are placed in novel environments (Fahlström et al., 2012). Analysis of the various parameters used to assess motivation to retrieve and consume a food reward failed to reveal a significant effect of either age or type of conditioned response (cf. Fig. 2). Overall, the results of this experiment show that motivation for rewarding foods is not altered by ageing.

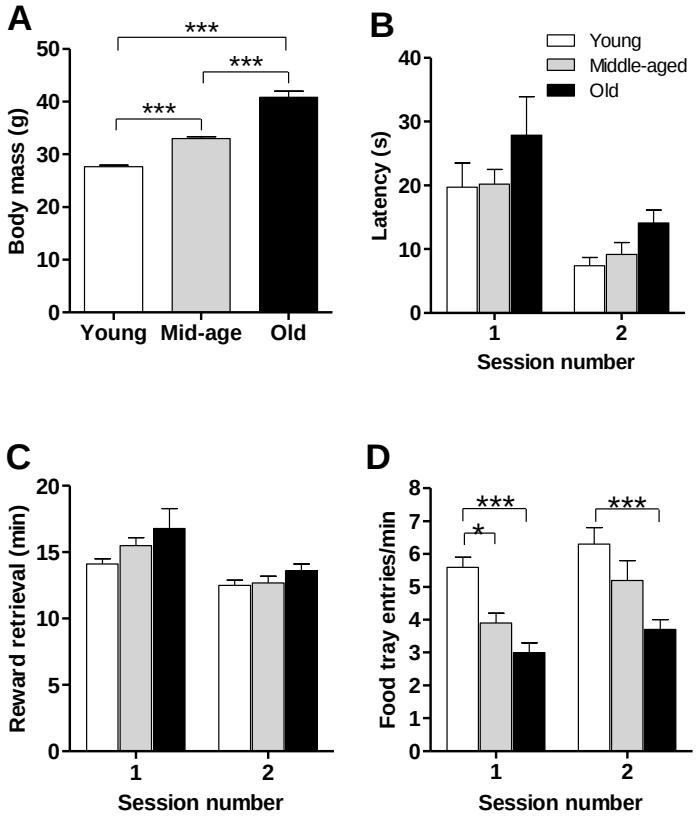

Figure 4.3 Motivation for food reward does not differ between young (n=15), middle-aged (n=16) and old (n=14) mice. Animals were rewarded with sweetened milk which is more rewarding than their standard food pellets. For each age group, initial body masses **(A)**, mean latency to approach the reward **(B)**, time taken to retrieve (and consume) the food reward **(C)**, and the number of food tray entries **(D)** are shown. Measurements were made over 2 sessions, with 15 reward deliveries in each. Results shown represent means ± SEM. * and * * * denote significant differences ($P < 0.05$ and $P < 0.001$, respectively) between the indicated pairs of data.

Hedonic preferences endure even in old age

Since food preference is an important factor in the development of eating patterns (Berridge and Kringelbach, 2013), examination of this parameter in differently-aged mice was undertaken to complement the data reported above. This was achieved by comparing the consumption of highly-rewarding (sweet and fatty), isocaloric liquid foods vs. standard solid chow over 24 h; animals previously had ad lib access to standard diet. The study was done in middle-aged and old mice, whose body weights were significantly different ($p < 0.001$, Fig. 4.4A).

The data in Figs. 4.4B and 4.4C depict the temporal patterns of consumption of sucrose and milk in the different age groups. Over 24 h, both groups consumed volumes of the liquid diets that exceeded their usual daily consumption of water (middle-aged: average water consumption = 2.7 ml/24 h; sucrose consumed = 10.5 ml/24, ($p < 0.001$) [vs. water]; milk consumed = 29.4 ml/24, ($p < 0.001$) [vs. water]; old: average water consumption = 2.3 ml/24 h; sucrose consumed =9.75 ml/24, ($p < 0.001$) [vs. water]; milk consumed = 19.5 ml/24, ($p < 0.001$) [vs. water]). Further, as shown in Figs. 4.4B and 4.4C, as compared to their middle-aged counterparts, the old mice consumed significantly less sucrose [$F_{1,99} = 9$; $p = 0.003$] and milk [$F_{1,99} = 31.5$; $p \leq 0.0001$] between 6 and 24 hours, although they showed similar rates of consumption during the first 6 h of testing; within group comparisons revealed that mice of both ages preferred milk over sucrose ($p < 0.001$) . Since the two liquid diets were isocaloric, these findings indicate a role of hedonic factors (smell, taste, texture) in the regulation of preference.

Computation of the total energy (calories/kg BW) derived from the combination of the sucrose and milk solutions and standard diet during the 24 h exposure to the food choice paradigm revealed that the energy intake of middle-aged mice was significantly higher than that of the old mice ($p < 0.001$; Fig. 4.4D). Interestingly, the relative amounts of energy derived from the highly palatable liquid diets did not differ between the two age groups (Fig. 4.4E); however, the younger mice derived relatively more energy from the standard diet ($p < 0.05$; Fig. 4.4E), consistent with their higher total energy intake.

In brief, these findings indicate that i) hedonic set-points are not changed during ageing in the mouse, and ii) that old mice can match their total daily calorific intake, derived from hedonically-loaded foods and a standard chow diet, to their (reduced) metabolic requirements just as efficiently as middle-aged animals.

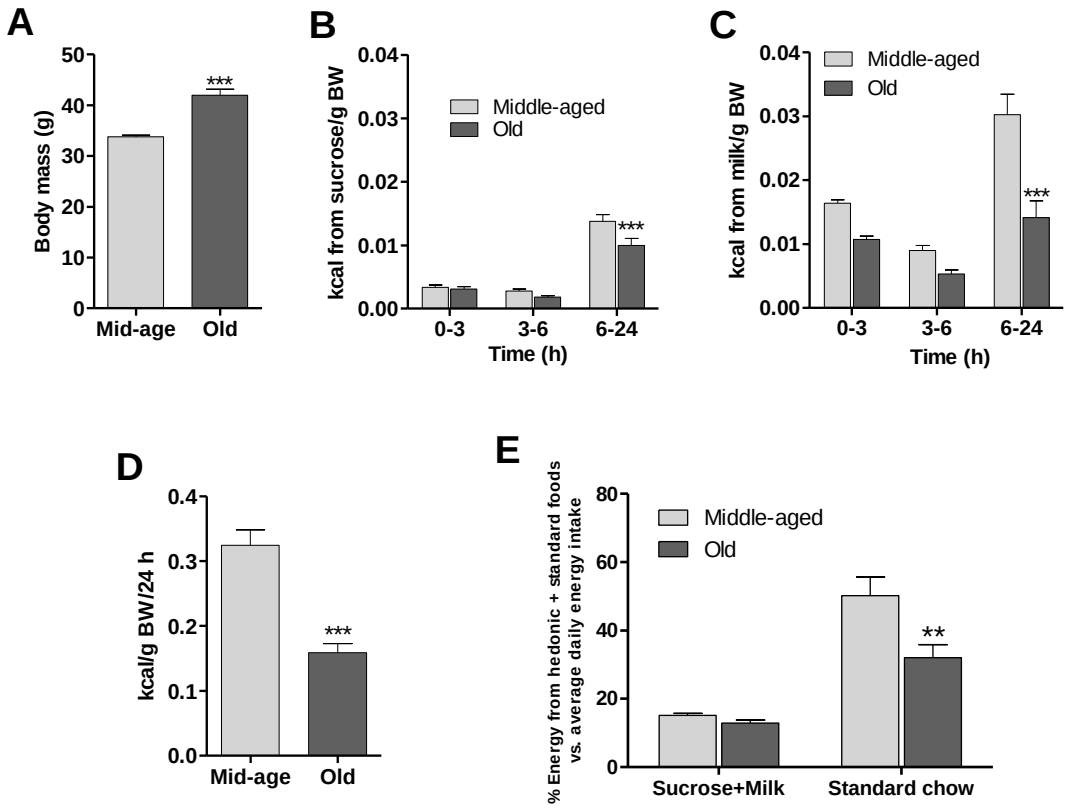

Figure 4.4 Hedonic preference test. The test compared consumption of two isocaloric palatable liquid-foods sucrose (15%) and milk (5% fat) in presence of unlimited amounts of standard chow diet by middle-aged (6 months, n = 20) and old (13 months, n = 15) mice. **A.** Initial body mass. **B-C.** Sucrose and milk consumption during different intervals over 24 h. **D.** Total energy intake (hedonic foods + standard chow diet) in 24 h, expressed relative to body mass. **E.** Relative (%) energy derived from either hedonic liquid foods (sucrose or milk) or standard chow during a 24 h test period vs. average daily energy derived from standard chow food during pre-test monitoring. All data are shown as means ± SEM. * * and * * * denote significant differences ($P < 0.05$ and $P < 0.001$, respectively) between middle-aged and old mice.

4.5 Discussion

The main finding of our experiments is that three essential components of feeding behaviour – conditioned learning, motivation and, ability to choose foods based on their hedonic properties – remain intact during ageing in the mouse. This evidence is important in light of the growing use of rodent models for understanding the mechanisms underlying human obesity and in particular, the search for the cause of mid-life obesity; the latter associates strongly with a spectrum of metabolic, neurological and psychiatric diseases during human ageing.

Although there is a large literature on age-induced impairments of declarative (explicit) learning and memory and on the processes and mechanisms responsible for such impairments (Driscoll and Sutherland, 2005; Hedden and Gabrieli, 2004), little is known about how ageing affects implicit memory. Implicit memory plays an important role in feeding behaviour, exemplified in the work by Schoenbaum et al. (2002); Roman et al. (1996) and Renteria et al. (2008); these authors reported that the acquisition and recall of conditioned responses (e.g. flavor-reward and odor-reward associations) are negatively influenced by ageing in rats. In contrast, we found that young, middle-aged and old mice do not differ in their performance in pavlovian (autoshaping) and operant conditioning paradigms, involving passive and active associative learning. Both, young mice (Harb and Almeida, 2014) and rats (Lomanowska et al 2011; Anderson et al 2013), develop sign-, goal- or intermediate-tracking behaviours during pavlovian conditioning. Similar conditioned learning responses were observed in the present study and, importantly, the distribution of response patterns was not a function of age, indicating that all the known features of conditioned learning are preserved during ageing.

The results from the two appetitive associative learning paradigms used in the present work are particularly relevant to the study of eating behaviour in the context of overweight and obesity. External cues have an important influence over the amount of food consumed by hungry and satiated rodents (Weingarten, 1983; Petrovich et al., 2007b). Appetitive conditioning has also been implicated in human feeding behaviour; for example, cues provided in the media and the general obesogenic environment that pervades modern societies strongly influence eating choices and eating patterns in children (Jansen et al., 2003; Halford et al., 2008; Birch and Anzman-Frasca, 2011), adolescents (Scully et al., 2012), and adults (Scully et al., 2009), independently of body mass status (Ferriday and Brunstrom, 2011; Ziauddeen et al., 2012b). The present findings demonstrate that subjects of any age can attribute incentive salience to food cues, thus placing them at risk for maladaptive behaviours, e.g.

overeating in absence of metabolic need. However, we previously found that sign-tracking conditioned responding – a possible surrogate of compulsive behaviour – does not predict propensity to overeat or develop obesity in the mouse (Harb and Almeida, 2014).

Besides the role of external cues in the conditioning of feeding behaviour, the inherent sensory (odor, visual appearance, taste and texture) and physical (energy content) properties of foods provide the motivational drive to seek food and to determine the amount of food ingested (Desmarchelier et al., 2013; Beeler et al., 2012; Rolls, 2010; Fernstrom et al., 2012; Mehiel and Bolles, 1988; Li et al., 2013). Since sensory processing is altered in many older humans, a phenomenon best exemplified by the so-called "anorexia of ageing" (Morley, 1997; Donini et al., 2003; de Boer et al., 2013), we compared the motivation to eat, including the consumption of foods with high hedonic value, in young, middle-aged and old mice. An experiment in which we measured the time taken to retrieve and consume a rewarding food revealed that although younger mice are more reactive than their older counterparts in terms of stimulus touches, motivation to eat remains stable throughout the lifespan of the mouse. This finding contrasts with that of an earlier report that older rats have reduced motivational drive (Frutos et al., 2012); however, the results of that study, obtained in the "incentive runway" test, may be more reflective of differences in locomotor speed (older rats were slower) and body mass and energy reserves (greater in aged animals) than of motivational drive per se.

Given that the capacity for associative learning and motivation to eat do not differ between mice of different ages and, that responses to the hedonic qualities of foods may be impaired in ageing humans (Morley, 1997), we examined whether the display of hedonic preference for palatable foods is altered in ageing mice in this study. Our experimental results show that young and old mice do not differ in their consumption of milk or sucrose during the first 6 h of parallel presentation of these hedonically-charged foods with standard chow diet. On the other hand, while the relative consumption rates of milk and sucrose did not differ between young and old mice over the 24 h of testing, the older animals consumed less of their standard diet. These observations show that ageing does not interfere with hedonic choice-making and, that aged mice retain their ability to adjust calorific intake to match their daily energy requirements; the lower caloric intake by older animals may be attributed to the fact that, as compared to young mice, they are less active (Ingram, 2000) and thus expend lower amounts of energy; at the same time, older mice have access to a larger energy depot (fat mass) as compared to their younger conspecifics (Hariri and Thibault, 2010). Overall, our findings are consistent with

those of Frutos et al. (2012) who also reported that young and old rats do not differ in their hedonic reactions to sucrose and corn oil and that older rats show more rapid signs of satiation.

The key findings of the present study are as follows: Firstly, we show that implicit (associative) memory for food cues remains intact in old mice. This is not surprising since feeding provides essential nutrients and is the main source of energy for all living organisms. However, given the predominant focus of research on the causes and mechanisms of age-related decline in explicit memory, the present results raise awareness of the need for exploration of whether other types of implicit memory might also be preserved during ageing. Secondly, our experiments indicate that ageing does not necessarily reduce proneness to maladaptive feeding behaviour; this is because motivation and responses to hedonic signals about food are retained throughout mouse ageing. Interestingly, however, hypofunction of the neuroanatomical and neurochemical substrates that mediate motivation and reward has been reported in aged humans that have high amounts of abdominal fat and/or a high body mass index (Green et al., 2011). Thirdly, in contrast to humans who are at risk of developing obesity because hedonic signals prevail over homeostatic control mechanisms, mice are apparently able to maintain a balance between energetic demand and conflicting hedonic stimuli. Although a highly-conserved behaviour, eating is a complex behaviour whose regulation and execution represents integration of energetic, metabolic, sensory, cognitive, and motor functions; further, implicit memory plays an important role in eating. Age-related impairments in explicit memory are well established; the results presented in this study show, for the first time to the best of our knowledge, that implicit memory is unaltered in ageing. Thus, the risk for older humans to be conditioned by food-related hedonic cues is likely to be greater than previously thought; this combined with age-related reductions in physical activity, also place them at risk for developing obesity and its associated disorders.

CHAPTER 5

General Discussion and

Research Perspectives

5.1 Current thoughts on behavioural mechanisms leading to overweight and obesity

The neurobiological and biobehavioural mechanisms of feeding behaviour are complex, being regulated through a multitude of endogenous peripheral and central signals (Fig. 1.1) as well as environmental sensory cues (detailed in §1.3). Improved understanding of the biological mechanisms underlying overeating is important in light of the rise in human obesity (Scully, 2014) and its repercussions on somatic (type 2 diabetes, cardiovascular disease and hypertension), and mental (mood, anxiety, Alzheimer's disease) diseases (Must et al., 1999; Haslam and James, 2005; Scott et al., 2008). It is generally agreed that obesity represents an imbalance between food intake and energy expenditure. Humans often tend to eat more calories contained in energy-dense foods that are rich in fat and sugars, at the cost of protein, complex carbohydrates and dietary fibre, because of their abundance, lower-cost and, importantly, their hedonic properties (Simpson and Raubenheimer, 2014).

One idea regarding the causes of obesity that gained popularity is that obesity is similar to addictive and substance abuse disorders (Volkow and O'Brien, 2007). This view mainly arises from the fact that there are some similarities in the neurochemical (dopaminergic imbalance) and cognitive/behavioural (compulsivity, loss of control, craving, feelings of guilt, unsuccessful attempts to cut the habit) processes involved in drug addiction and excessive eating. However, the idea that eating can turn into an addictive behaviour has provoked contentious debates in recent years (Wang et al., 2001; Avena et al., 2005; Epstein and Shaham, 2010; Peters, 2011b; Ziauddeen et al., 2012). For example, many of the obesity-addiction arguments are based on sugar binging in laboratory rodents even though there is no clear correlation between food binging and obesity in humans (Wonderlich et al., 2009).

Other researchers have proposed obesity results from a "reward deficiency syndrome", characterized by an insufficiency of usual feelings of satisfaction (Blum et al., 2000), where overweight people cannot derive sufficient pleasure from hedonic foods, due to a dysfunction in the "brain reward cascade", namely in the complex interaction among neurotransmitters (Fig. 1.5, 1.6). The dopamine imbalance hypothesis as the main cause of reward deficiency syndrome and obesity has not received unequivocal support (Ziauddeen et al., 2012; Volkow et al., 2013; Ziauddeen and Fletcher, 2013).

Newer perspectives emerged from the suggestion that hedonic hunger or the desire to eat purely for the sake of pleasure, triggers overeating and weight gain. This theory posits that the initiation

of feeding in absence of physiological hunger, induced on one hand by the palatability of foods and, on the other, by appetitive conditioning to an abundant environmental cues causes uncontrollable desires and difficulty to resist conditioned feeding stimuli, this means overriding metabolic needs and physiological signals of satiety (Weingarten, 1983; Jansen et al., 2003; Petrovich et al., 2007b; Halford et al., 2008).

The studies reported in this thesis have attempted to contribute to extend our understanding of feeding behaviour by analyzing key elements of feeding behaviour, namely, associative learning, hedonic preference and motivation. The experiments were done in healthy, overweight, obese, young and aged mice, a species commonly used to examine appetitive behaviour in the context of the regulation of human eating behaviour. As discussed below, the results of our experiments

- show that food cues can elicit conditioned learning responses throughout the lifespan (*Chapters 2* and *4*)
- challenge - the addiction theory of overeating (*Chapter 2*);
- challenge the reward deficit syndrome (*Chapter 3*);
- support the importance of hedonic hunger, exemplified by the vulnerability to develop conditioned responses to food-related cues and the tendency to overconsume hedonic foods as a major factor behind the overriding of metabolic signals and needs (*Chapter 2* and *4*);
- question the generalization of observations in laboratory rodents to humans (*Chapter 3*).

5.2 Phenotypes displayed in pavlovian conditioning do not predict susceptibility to obesity and drug addiction

The work described in *Chapter 2* of this thesis used the Pavlovian conditioning paradigm to study the propensity of sign-tracker (ST) mice to develop compulsive overeating. It was inspired by numerous articles (Flagel et al., 2009; Robinson and Flagel, 2009; Lomanowska et al., 2011; Saunders and Robinson, 2011; Meyer et al., 2012; Yager and Robinson, 2010, 2013) suggesting that environmental food and drug cues acquire greater incentive stimulus properties (serve as more effective conditioned

reinforcers) in ST *vs.* goal-tracker (GT) animals. These authors hypothesized that ST will be more susceptible to develop compulsive behaviours such as drug addiction and overeating.

In *Chapter 2* we show that mice, like other species, are capable of acquiring different conditioned responses (CR) in an appetitive pavlovian conditioning paradigm, namely, ST (persistent responding to the conditioned stimulus), GT (conditioned response directed to the unconditioned stimulus, the food tray), and intermediate tracking (IT, alternation between conditioned and unconditioned stimuli). These CR represent robust associative learning, which can be sustained throughout the lifespan, since segregated conditioned responses (ST, GT and IT) were obtained with food reinforcers of differing caloric content and palatability in mice of different ages (*Chapters 2* and *4*). Further, results in *Chapter 2* demonstrate that ST behaviour is not predictive of overeating since ST animals do not differ from GT mice in terms of their behavioural responses to novelty or emotionality, and food reward-seeking and consumption behaviour. In addition, ST and GT mice do not differ in their food consumption and body fat accumulation when placed on an obesogenic high fat diet (HFD) for a long period.

In contrast to our observations, other studies reported greater impulsivity in ST animals (Lovic et al., 2011) and that ST animals engage in more novelty-seeking behaviour (Beckmann et al., 2011) and have poorer executive control over behaviour (Paolone et al., 2013). All of these behaviours may be considered to be "risk factors" for addictive behaviour. Importantly, however, other recent work revealed that GT rats may also show susceptibility to drugs of abuse insofar that they are more reactive than ST rats to context-dependent conditioning and show more drug seeking behaviours (Saunders and Robinson, 2012). Accordingly, T.E. Robinson, a pioneer of the original work describing a correlation between ST and addictive behaviours recently wrote: "...STs are not more susceptible to addiction than GTs, but that for different individuals there are *different pathways to addiction*...." (Robinson et al., 2014).

Together, the above findings evince that ST in pavlovian conditioning does not mark vulnerability to compulsive behaviour; the discovery of phenotypes predictable of susceptibility and predisposition to obesity and drug addiction may therefore be more difficult than assumed.

5.3 Learning ability, motivation and hedonic preference for food in overweight and obese mice

The work in *Chapter 3* involved testing male mice that differed in body weight (normal, overweight/moderately obese and obese mice generated by maintenance on diets of differing obesogenic potential) to acquire two appetitive-associative learning tasks (pavlovian and operant conditioning), motivation and hedonic preference.

Many studies have investigated the role of increased body fat and high fat diet intake on mood and cognition in humans) and rodents ((Sellbom and Gunstad, 2012; Lakhan and Kirchgessner, 2013), albeit with inconsistent conclusions. While some authors found an association between greater body mass and poor performance in global cognitive, (Elias et al., 2003; Whitmer et al., 2005), memory and language, (Gunstad et al., 2010) as well as increased risk of dementia and Alzheimer disease (Jagust et al., 2005; Kivipelto et al., 2005), others hypothesized that weight loss, rather than weight gain, in old age reduces functional abilities and poses a risk for Alzheimer's disease (Barrett-Connor et al., 1996; Stewart et al,. 2005). Variations in paradigms and end-points measured account for the differences in clinical reports.

The relationship between body mass and cognitive and affective behaviour is also unclear in rodents. While many studies have reported that obesity impairs spatial learning (hippocampus-dependent) and memory (Ross et al., 2009; Heyward et al., 2012; Beilharz et al., 2014) in mice (Farr et al., 2008; Morrison et al., 2010) and rats (Molteni et al., 2002; Wu et al., 2004; Jurdak et al., 2008; Stranahan et al., 2008) as well as synaptic plasticity in the hippocampus, others failed to detect significant declines in performance in the Morris water maze (Mielke et al., 2006; McNeilly et al., 2011; Pancani et al., 2013), object location memory and Y-maze tasks (Valladolid-Acebes et al., 2013). Further illustrating the discrepancies in the rodent literature are the fact that whereas contextual fear conditioning memory was shown to be negatively impacted upon in obese mice (Heyward et al., 2012), but other authors did not see this impairment (Hwang et al., 2010).

The results obtained here in tests of associative learning indicated poorer learning of appetitive conditioning tasks by overweight and obese mice (Fig. 3.1, 3.2). However, our analysis revealed that the poorer performance was not due to a decline in cognitive abilities but rather to a lack of motivation toward the reinforcer (food) and the lower energetic needs of animals with high body mass (Fig. 3.3,

3.4A, 3.4B). Briefly, overweight and obese mice exhibit proportionally lower motivation to learn and to consume the food reward.

Our findings contrast with interpretation from other studies in which food was used as a reinforcer to test learning and memory in rodents on high fat diets. For example, short- (Murray et al., 2009) and long- (Greenwood and Winocur, 1990;Valladolid-Acebes et al., 2011) term exposure to HFD was found to induce a deficit in spatial and working memory in the radial arm maze when palatable foods served as reward. Similarly, procedural learning was impaired, when food was rewarded in an operant paradigm (Greenwood and Winocur, 1990; Mielke et al. 2006; Farr et al. 2008; McNeilly et al. 2011). The present experiments (Fig. 3.3) clearly strongly suggest that these types of memory cannot be studied in appetitive paradigms, mainly because overweight/obese animals have reduced appetitive need (wanting) and interest in food reinforcers; it is important to note that the motivation task used by us did not require learning. We conclude that animals with higher levels of adiposity work less for food rewards because of their lower actual energetic demands. Metabolic signals from adipose tissue, such as leptin, which are known to provide the brain with information about energy homeostasis and reward/motivation (Figlewicz et al., 2006; Peters and Langemann, 2009; Kanoski et al., 2014) may play a role here, as discussed in *Chapter 1*.

Consistent with our findings, numerous studies in rodents have suggested that the rewarding valence of food is strongly modulated by nutritional status (hungry, satiated) and by metabolic regulators (such as ghrelin, leptin, insulin) (Cabanac and Duclaux, 1970; Figlewicz et al., 2006, 2007; De Jonghe et al., 2007; Figlewicz and Benoit, 2009). Hungry animals show enhanced motivation to work for food (Figlewicz et al., 2007) and, oppositely, satiety modulates hedonic reactivity to palatable foods (Berridge, 1991). Moreover, besides being able to modulate food- and non-food- related learning and memory (Harvey, 2007; McNay, 2007) and the sensitivity of taste receptors (Peters et al., 2006), leptin and ghrelin (see *Chapter 1*) can exert a powerful influence on the visual perception of food in the human environment; while leptin diminishes appetite (Uher et al., 2006; Farooqi et al., 2007), ghrelin enhances feelings of hunger and appetite (Schmid et al., 2005). These findings have been confirmed in functional neuroimaging studies that show that neural activity in brain regions such as the amygdala and OFC, which are involved in valence attribution to food varies according to nutritional status; for example, Small et al., (2001) showed that a food stimulus that is rewarding and induces feelings of pleasure during the hungry state can trigger aversion under conditions of satiety.

Much research into the behavioural basis of obesity has centered around dopamine (DA) transmission in view of earlier-discussed ideas that obesity results from food addiction or a reward deficiency syndrome (Volkow and O'Brien, 2007; Volkow et al., 2013). On the one hand, correlational studies that support the view that obesity reflects a state of reward deficiency describe decreased DA signaling and attenuated mesolimbic DA turnover and DA receptor expression in the N.acc (Alsiö et al., 2010; Geiger et al., 2009; Davis et al., 2008) of obese rats; in humans, obesity is associated with decreased striatal responses to food intake (Stice et., 2008). There are also reports of reduced striatal dopaminergic D2 receptors in obese humans and rats (Wang et al., 2001; Hajnal et al., 2008; Volkow et al., 2008b); the latter, suggesting increased DA release, has been interpreted as the cause of overeating (see Comings et al., 1993; Epstein et al., 2002; Annerbrink et al., 2008). On the other hand, other reports show that obese people display increased pleasure to foods that are rich in fats and sugars (Saelens and Epstein 1996; Rissanen et al., 2002; Davis et al., 2004) and, greater striatal response to visual food-related cues than lean subjects (Rothemund et al., 2007; Stoeckel et al., 2008; Stice et al., 2010).

Given that all drugs of abuse induce behavioural sensitization following sensitization of monoaminergic pathways, it was interesting that the present studies observed that control, overweight and obese mice cross-sensitize to behaviour morphine-induced hyperlocomotion (Fig. 2.5) in a similar manner. The fact that overweight and as obese animals do not display behavioural sensitization indicates intact monoaminergic (DA, norepinephrine, serotonin) systems (see Fig. 1.5), suggesting that obesity is neither a consequence nor a cause of imbalanced DA release, i.e. unlike to the situation in drug addiction (Volkow et al., 2013a). We also showed that morphine-sensitized obese mice do not over-consume highly rewarding sucrose (solution) (Fig. 2.5), endorsing the view that even though the rewarding and motivational effects of both food and drugs of abuse are mediated by similar neurochemical mechanisms, obesity and drug addiction represent distinct states that result from the summation of disparate dysfunctional input and output pathways.

As mentioned previously, animals with lower fat depots display increased motivation and consumption rates of palatable foods than their counterparts with higher body mass (*Chapter 3*). However, as shown in (Fig. 3.4, 3.5), overweight and obese mice do not show signs of reward deficiency syndrome when exposed to two isocaloric hedonic foods (sucrose and milk) in the presence of their respective maintenance diets; rather, these animals appear to have lower energetic needs (Fig. 3.4B) and their motivation to work for foods that are less-energy rewarding (than their maintenance diets) are

accordingly reduced (Fig. 3.4E). Interestingly, although control mice consume more of the sucrose and milk solutions than their overweight and obese counterparts, none of the groups differ in terms of caloric intake in a test of hedonic preference. Briefly, all groups maintained their metabolic *status quo* through differential consumption (amount) of the different types of food presented (recall, controls were maintained on standard chow which is less rewarding than the high-fat/carbohydrate diets given to overweight and obese mice) (see Fig 3.6).

Together, the above sets of evidence introduces an important point with respect to interpretation of results on the link between the behavioural elements controlling appetite in animals of different body masses: the observation that obese animals (maintained on energy-rich, high fat or high sugar diets) ingest fewer calories from less-hedonic foods, as compared to animals of lower body mass, is not necessarily indicative of a reward deficiency syndrome but rather, a lower preference for foods that are less less-energy dense than their maintenance (high fat/sugar) diets. The data shown in Fig. 3.4B reiterate the importance of fat stores and energy abundance in the regulation of feeding via peripheral signaling pathways to brain regions involved in motivation and reward, and ultimately energy homeostasis, as described in *Chapter 1*.

5.4 Influence of ageing on learning, motivation and hedonic preference for food

A major focus of research in the field of obesity has been on the development of overweight and obesity in children and adolescents. This is because youth are highly susceptible to appetitive conditioning and easily develop maladaptive behaviours, but has been at the cost of research on older individuals (Halford et al., 2004, 2008; Boyland et al., 2011). The latter are, on the other hand, very susceptible to developing obesity-related diseases such as type 2 diabetes, cardiovascular disease, immune disorders and brain disorders such as depression, anxiety and impaired cognition, including Alzheimer's disease (Elias et al., 2003; Mathus-Vliegen et al., 2005; Fitzpatrick et al., 2009; Gunstad et al., 2010; Topic et al., 2013).

Chapter 4 addressed the question of whether learning, motivation and food preference are altered during ageing in mice. To that end, we compared young, middle-aged and old mice

A key result was that, the three essential components of feeding behaviour, appetitive learning (pavlovian and operant conditioning), motivation and hedonic preference, are not affected by ageing in male mice. Specifically, we observed that young, middle-aged and old mice develop similar pavlovian conditioned responses, segregating into ST, GT and IT, and show identical responses in an operant conditioning paradigm; in both tests of learning, the rewarding stimulus was a food. Further, we found that mice of different ages do not differ in their motivation to retrieve and consume highly-rewarding foods. Interestingly, though, when highly-palatable foods were presented alongside a standard diet, aged mice were apparently able to adjust their ingestion patterns so as to match their usual daily caloric intake by consuming similar amounts of the hedonically-loaded foods and less the standard chow they were maintained on. These findings imply that the brain regions governing the regulation of appetite (and energy homeostasis) are sustained during ageing, a reflection of the fact that eating is a highly-conserved behaviour that is essential for survival.

These data show that vulnerability to conditioning by food-related sensory stimuli exists throughout the organism's lifetime, and that overeating and obesity can develop at any age.

5.5 Research perspectives – ongoing and future

The results of experiments conducted in this thesis alert us to the shortcomings of assuming that findings regarding eating behaviour in laboratory rodents can be extrapolated to humans, and vice versa. The main work here focused on the fact that a large part of feeding behaviour is based on conditioned learning. Unlike mice, humans are exposed to an abundance of potential food-related cues and have a wide choice of (healthy and unhealthy) diets and dietary components. Thus, the challenge that humans face to balance physiological *vs.* hedonic signals is much greater than that which laboratory rodents are confronted with.

The present work was carried out exclusively in male animals. However, many non-sexual behaviours, including learning and memory and emotional state, differ between the sexes in both rodents and women (Dalla and Shors, 2009); many of these behaviours are further modulated by the cyclical fluctuations in ovarian hormone secretion. Even though direct translation from mouse to human may not always be possible, it is important to note that there are major sex differences in the

incidence of neuropsychiatric diseases; for example, major depression (Nolen-Hoeksema et al., 1999), anxiety disorders (Pigott, 2003) and Alzheimer's disease (Viña and Lloret, 2010) occur more commonly in women than in men. Moreover, risk for these diseases is increased in obese individuals (Hrabosky and Thomas, 2008; Fitzpatrick et al., 2009) and at least one study has shown that obesity retards the therapeutic efficacy of psychoactive drugs (Kloiber et al., 2007). Notwithstanding the caveats regarding rodent-human comparisons, it will be interesting to conduct experiments similar to those described in this thesis in female mice, especially in view of the fact that human females are more susceptible to eating disorders.

Besides gender, motivational drive for food is likely influenced by emotional state. In animals, this can be easily studied using well-established stress paradigms, ranging from chronic exposure to multiple stressors to social stressors (e.g. resident-intruder paradigms). On the other side of the coin, it would also be interesting to learn about how appetitive conditioning changes in set-ups, such as environmental enrichment, which are known to protect individuals from the negative impact of stress on brain and behaviour. Such knowledge could contribute to the neurobiological basis of preventative strategies in humans. Research connecting feeding behaviour with stress is also important because of the known impact of stress on the regulation of body weight. While many stressors, in particular those with a strong psychological component, lead to body weight loss despite overeating of energy-dense ("comfort") foods in many instances (Oliver et al., 2000; Dallman et al., 2003; Pecoraro et al., 2004; Tomiyama et al., 2011), stress is also known to make individuals vulnerable to excess weight gain and type 2 diabetes (Bartolomucci et al., 2009; Dallman, 2010). These outcomes appear to be paradoxical because glucocorticoids – the main adrenocortical hormones released during stress – are, by definition, catabolic agents. The apparent paradox may be resolved by improved insight into interactions between glucocorticoids and the various peptidergic and neurotransmitter regulators of appetite and metabolism, as well as interactions between glucocorticoids (and their metabolism) and the complex biochemistry of fat depots which not only release appetite-regulating signals such as leptin, but also produce pro- and anti-inflammatory cytokines that act on the growth of fat cells but also at different peripheral (e.g. pancreas, liver) and central (e.g. hypothalamus) levels to control energy homeostasis.

One of the best examples of a stress-related disorder is major depression (Kendler et al., 2001), where animal models continue to play a significant role in research. Among others, researchers

commonly employ the sucrose preference test (SPT) as a measure of anhedonia, a cardinal symptom in human depression. However, the SPT is confounded by a number of biases. It is usually conducted in animals that have been deprived of food and water for 24 h, and preference is gauged by the animal's choice of a low percentage of sucrose (1% to 2.5%) *vs.* water; often the test is carried out in the absence of other sources of energy (e.g. standard diet). The results presented in *Chapter 3* of this thesis raise questions as to the complete validity of such a testing regimen; while the removal of solid diets and prior food/water deprivation are supposed to increase motivation for the sucrose solution, one could argue that the energy in 1-2.5% sucrose will not provide sufficient (energy-seeking) motivation for the animals, thus potentially leading to false results and interpretations. In other words, the SPT has a strong motivation component (decreased in depression) that is sometimes nevertheless overlooked. The motivation and hedonic preference tests described in *Chapters 2-4* (described in detail, for rats, in *Annex I*) may help improve current tests of depressive-like behaviour in rodents. Briefly, the hedonic preference test is based on choice between two isocaloric foods that differ in palatability (hedonic properties) over 24 h; preference for these foods is monitored in satiated and hunger states. Preliminary data showed that rats exposed to chronic mild stress (CMS, an established paradigm for inducing depression-like behaviour) are neither anhedonic (Fig. A1, *Annex I*) nor had a motivational deficit (Fig. A2-A4, *Annex I*); rather, CMS-treated rats displayed more sucrose preference than their non-stressed counterparts.

Results presented in *Chapters 2 and 3* suggest that, at least in mice, feeding is not an addictive behaviour. We propose that although drug addiction and obesity may share common limbic reward and motivation circuits, the two conditions are likely to be distinct pathological entities. Here, it is worth considering that obese subjects can show an attenuated behaviour towards food rewards, a phenomenon that is reversible after induction of hunger by food restriction (Shin et al., 2011). In contrast, addicted subjects attribute high salience toward drug and substance rewards and cues (incentive salience); this is a behavioural disinhibition that is difficult to reverse (Robinson and Berridge, 1993). Further experimentation to reappraise the still-popular view by that obesity results from an addiction to food is needed. If "food addiction" and drug addiction have identical underpinnings, one would expect cross-sensitization between food and drug rewards. The results presented in *Chapter 2* demonstrated that there is no cross-sensitization between food and morphine, the prototypic drug of abuse.

The "addiction hypothesis of obesity" has been largely driven by correlates of altered DA transmission during feeding in lean and obese subjects (Wang et al., 2001,2004). As described in *Chapter 1* (see Fig. 1.5), many neurotransmitters (e.g. NE, 5-HT) and neuromodulators (e.g. orexin, leptin) contribute to the control of appetite and body weight; moreover, many of them are implicated in the control of emotion, reward, learning and memory, attention, alertness, motivated behaviour, and even addictive behaviour. Consideration of how these various input-output peripheral and central signals are integrated will be essential for better understanding of commonalities and differences in the neurochemical basis of overeating and addictive behaviour.

Studies such as those proposed above will also throw light on the question of whether, overeating represents a reward deficiency syndrome as has been proposed to occur in substance abusers and drug users (Comings and Blum, 2000). The reward sensitivity reduction hypothesis has been applied to overeating behaviour on the basis of experiments showing that obese rats acquire operant tasks in which the reinforcer is a rewarding food or drug of abuse (Carroll and Lac, 1998; la Fleur et al., 2007; Wellman et al., 2007). However, it may be argued that obese animals are fully rewarded by their obesogenic diets and are therefore not motivated by additional food rewards. In fact, it has been shown that the animals self-restricted their food consumption when the obesogenic diets were exchanged for standard diets; this response is sometimes falsely considered as a craving behaviour, resembling the addiction-like adaptive response to drugs of abuse (la Fleur et al., 2007; Pickering et al., 2009; Johnson and Kenny, 2010). Future studies addressing reward deficiency and addiction in obesity should consider the importance of "hunger" which is operationally synonymous with "energy need".

5.6 Societal implications of this work

Genetic predisposition (Bouchard, 2007) and perinatal programming (Pico and Palou, 2013) are important in biological determinants of obesity, but conditioning may be the single most important environmental factor that causes overeating and therefore obesity. Laboratory animals appear to be capable of regulating their eating behaviour to ensure that their essential metabolic needs are satisfied. In contrast, hedonic hunger which overrides metabolic signals appears to be the unwanted side of

industrialization and wealth which have brought both abundant and affordable food supplies and food choices. Industrialized societies are also exposed to a multitude of food-related cues, widely propagated, often with misleading or false claims, in advertisements; many of the latter are specifically designed for particular target audiences of different ages, sexes, and health, educational and socio-economic status, and work by conditioning.

Just as advertising has effectively worked to promote excessive desires and overeating, educational programmes – that also depend on conditioned learning – can be used to raise awareness of the long-term health risks of obesity and to motivate healthful eating; evidently, the importance of healthy eating habits has not penetrated society to a sufficient extent (Birch and Anzman-Frasca, 2011). Such preventative measures are already being put into field practice and target children and parents (Corsini et al., 2011; de Droog et al., 2011; Foltz et al., 2012; Dickens and Ogde, 2014). A slightly different approach, recent regulations approved by the European Parliament (Amended Regulation (EU) No 1308/2013 and Regulation (EU) No 1306/2013) to promote healthy foods (e.g. fruit, vegetables, milk) in schools also subtly exploits conditioning. Many children are today used to consuming convenience foods that lack nutritional value or that may actually include unhealthy components, and many find certain taste in healthy foods averse. The best examples of such foods are members of the *Brassicaceae* family (cabbage, sprouts, kale, and broccoli) which contain thiouracils (Bell and Tepper, 2006). Such children (but also adults) are often referred to as "supertasters"; such people are now known to carry a variant of the taste receptor gene *TAS2R38*, but this does not necessarily mean they should be allowed to avoid certain or any vegetables. Watching peers enjoy such foods or educating schools and parents about ways to cook these vegetables so as to mask their bitterness could result in greater consumption of these and other healthy foods. This is also important because early-formed eating habits are maintained throughout life. However, sensory systems, including taste, are highly plastic and even adaptive; for example, taste sensitivity changes during development and ageing: thus, a child who finds coffee aversive may find it highly pleasurable from the teen years onwards. Comparable changes in eating habits can be introduced through experience and/or peer, parental, educator and medical encouragement.

Our conclusion (albeit based on studies in mice) that, an essential behaviour like feeding cannot become an addiction, may have implications for dealing with current obese members of Society. Addicted subjects are stigmatized because sometimes they are seen as individuals who "refuse to

control their habits". Distinction between addictive disorders and eating disorders will at least destigmatize the latter and motivate the affected individuals to exert greater behavioural control over their eating patterns. While increasing caloric expenditure (e.g. through physical activity) will offset some of the excess calories consumed, learning to eat less and better is certainly the more economic and healthy approach.

Dieting (reducing caloric intake, or restricting the intake of certain food components) is often seen as the only way to reduce body mass. Such measures usually have only transient effects (Mann et al., 2007), with the so-called "yo-yo" effect potentially causing greater distress and reduced motivation to maintain the new dietary style. According to Peter's "selfish brain" theory (Peters et al., 2011c), this may be because modern dieting may be equated with famine and hunger in human history. Hunger states will alert the brain to an impending danger (reduced energy), which will in turn, switch the individual's behaviour and physiology into a mode that conserves energy by reducing metabolic activity. In parallel, the brain will become more attentive to and aroused by food cues, motivation to eat will increase, and energy utilization by peripheral organs will be minimized so as to regain lost body weight (Wang et al., 2006). Even though the "selfish brain" theory remains just a theory, it is attractive in the context of weight reduction strategies insofar that it suggests that encouragement of (or development of means to) sense metabolic signals and needs might be a more effective, sustainable and healthier way to achieve a target body mass that may prevent or delay the onset of obesity-associated chronic disease.

ANNEX I

Hedonic preference test

Method: Rats were subjected to chronic mild stress (CMS) to induce depression-like behaviour as described in *Annex* II. The hedonic preference test was performed in animals' home cages.

Rats were presented with two highly-rewarding isocaloric (64 Kcal) drinking solutions (15% sucrose *vs.* milk whose fat content was 3.5%). Animals had ad lib access to standard chow and water throughout. The volumes of sucrose and milk solutions consumed within 24 h were measured. In brief, this protocol allowed assessment of the hedonic preference of the liquid diets, independently of the animals' state of satiety or energy needs. In a second step, rats were food-deprived for 24 h and allowed to choose between the milk and sucrose solutions; testing was done in the home-cage but animals did not have access to their normal chow. This design allowed discrimination between hedonic preference vs. energy.

Results: Glucocorticoids are catabolic and stressful episodes can lead to overeating of comfort (high hedonic value) foods (Dallman, 2010). We therefore tested animals' preference for two isocaloric foods (sucrose *vs.* milk) in rats exposed to CMS in their daily periods of activity (light) or rest (light); since the CMS procedure led to body weight losses, a recovery interval was included to allow all animals to reach the same body mass (Fig. A.1C). While animals had *ad lib* access to standard chow, those rats that had received CMS in the light phase consumed significantly more sucrose and milk over 24 h than animals exposed to CMS during the dark phase (***P<0.001) (Fig. A.1A); these between-group differences persisted when solid food was not available (***P<0.001) (Fig. A.1B). In both tests, we observed that animals preferred the sucrose drink over the milk solution, irrespective of their energetic state (*ad lib* food *vs.* standard food deprivation) (Fig. A.1D).

Our results convincingly show that animals given CMS during the inactive (light) phase consume more sucrose solution than those given CMS during the daily period of darkness. This result is consistent with the finding that glucocorticoid secretion is disturbed in rats exposed to CMS in the light phase. We also show that the increased consumption of sucrose is related to disrupted energy metabolism since all animals had the same body weight at time of testing.

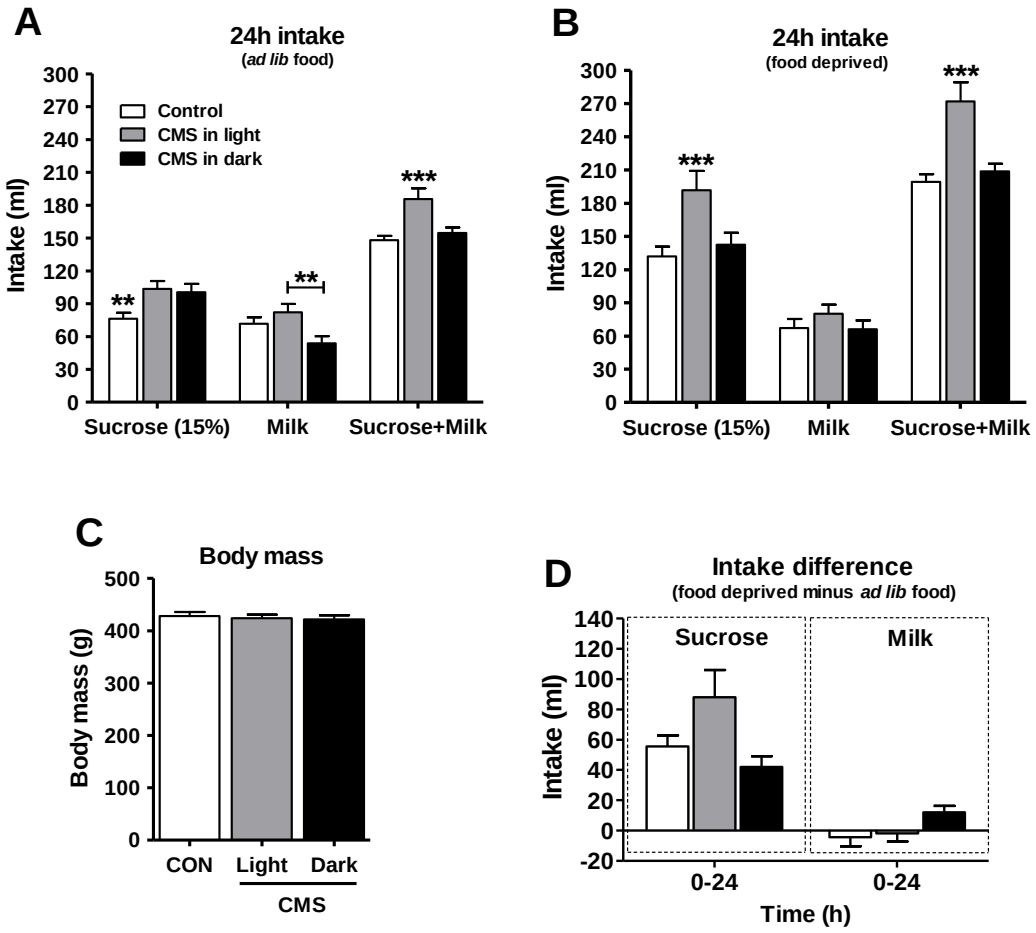

Figure A.1 Hedonic preference test between two isocaloric drinking solutions (15% sucrose *VS.* milk whose fat content was 3.5%) over a period of 24 h, in presence of their standard chow **(A)** or in absence (food-deprived) for 24 h **(B)**. **C)** Body mass after recovery from CMS. **D)** The amount of sucrose and milk ingested in food deprived test subtracted from the *ad lib* test. (n=14 for the control group, n=16 for each CMS group; ** P < 0.01. *** P < 0.001. Data presented as mean ± SEM).

Motivation test:

Apparatus: The motivation task was performed in automated chambers (TSE). A visual stimulus was presented in the stimulus hole at the front part of the box. Responses into the hole were monitored with infrared sensors – correct responses were reinforced by food reward (dustless sucrose pellets; 45 mg) delivery in the food tray on the opposite wall. ***Habituation:*** Rats were habituated to the experimental room and to the experimenter by handling each rat for at least 1 week before experiment starts. Each rat was placed 10 minutes in the box, during which the central light is off, but the stimuli hole and the food tray are on. This time was given to the animals to discover the boxes and to find and to consume the 8-10 pellets placed inside the stimuli hole and the food tray. During this habituation week rats were placed on a caloric-restriction schedule, they had 1 hour of ad libitum feeding per day, to induce 10-15% loss of original body weight, and this loss was maintained during all the testing period. Water was always provided *ad libitum.* ***2 days of training:*** Each rat performed 1 session of 60 trials per day. In order to receive the food reward, the animals should nosepoke the visual stimulus after every presentation. After the nosepoke, the visual light is turned off. And once the pellet is collected from the food tray, an interval eating time is given to consume the pellet. The onset of the next trial (presentation of the visual stimuli) is after 3 seconds from the eating interval. The visual stimuli is presented for maximum time of 1 minute, if the rat does not approach and nosepoke the stimuli hole the trial will be considered as omission and the new trial will start again after turning on the central light for 5 seconds. The data only of the second session is collected; all the animals that reach the criteria of finishing the 60 trials at a maximum time of 45 minutes per session are included in the analysis. ***Testing day:*** It is a one day session performed directly after the training. The same rules applied during the training will be also applied in this session. The only difference is that the 60 trials are divided in 4 blocks of 15 trials each, in which the visual stimuli is presented at different intervals after the reward consumption: 3 seconds first 15 trials / 6 seconds second 15 trials / 12 seconds third 15 trials / 24 seconds forth 15 trials. Only the animals that reach the criteria of finishing minimum 50 out of 60 trials at a maximum time of 45 minutes per session are included in the analysis. ***Measurements:*** Time to finish session - Number of trials per session – Nose poke correct – Omission - Latency to feed - Response latency – Perseveration – Premature response - Premature response time.

Results: Stressed animals showed similar motivation compared to the control ones.

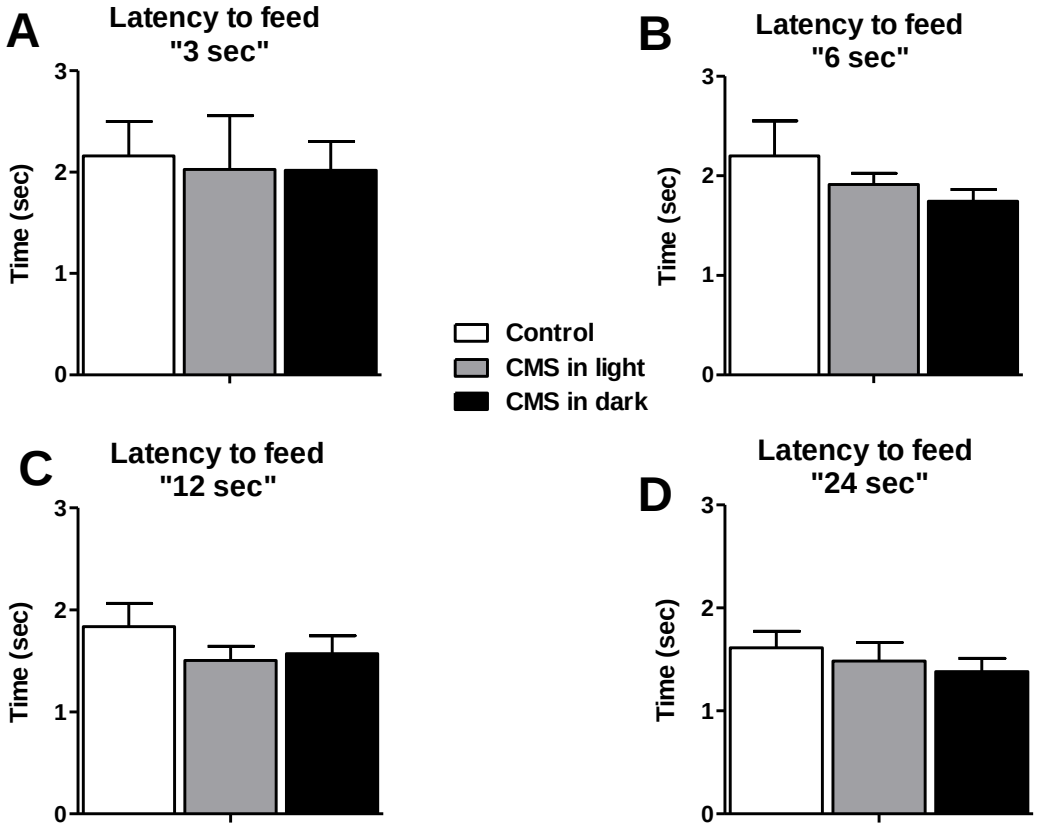

Figure A.2 Average latency to retrieve the food reward during 60 trials. The visual stimuli was presented at different intervals after the reward consumption: **A)** 3 seconds first 15 trials, **B)** 6 seconds second 15 trials, **C)** 12 seconds third 15 trials, **D)** 24 seconds forth 15 trials. Only animals that reached the criteria of finishing minimum 50 out of 60 trials at a maximum time of 45 minutes per session were included in the analysis.

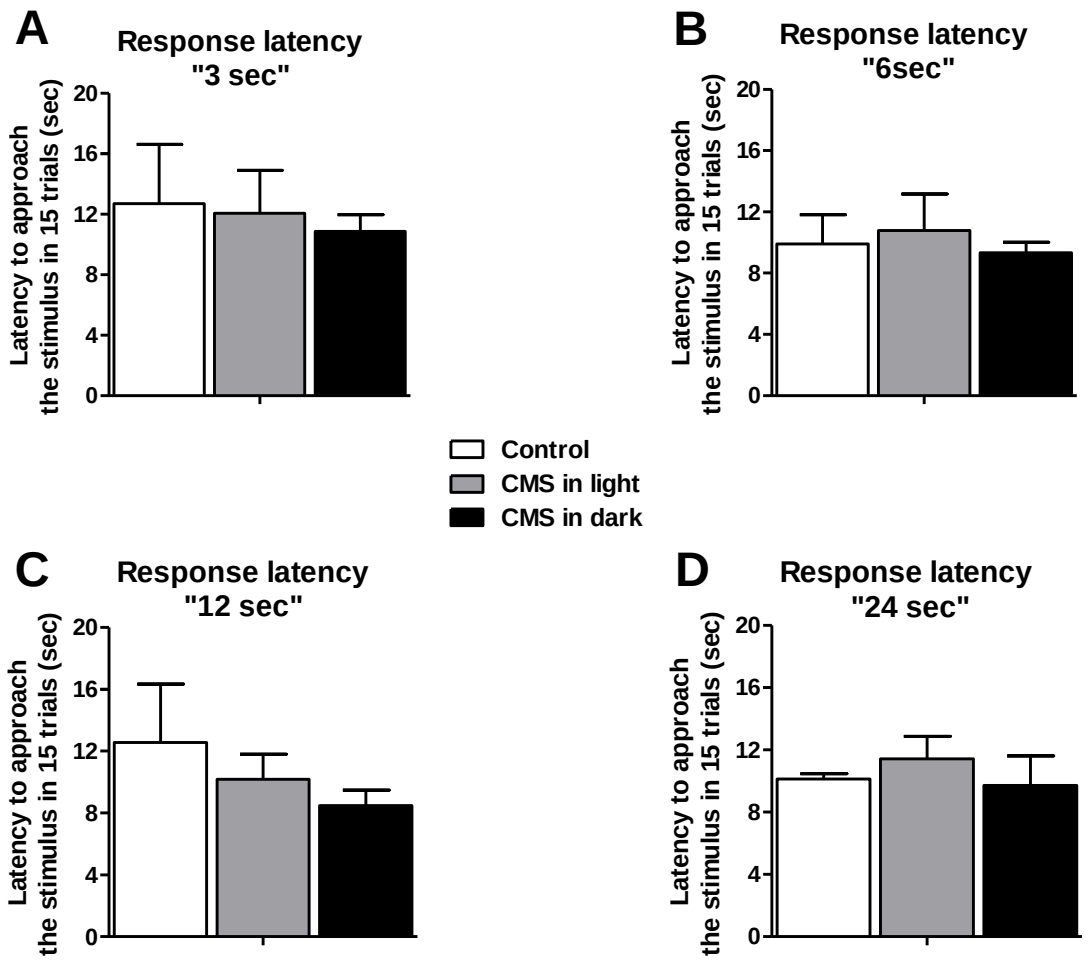

Figure A.3 Average latency to approach the visual stimulus every 15 trials (15 trials x 4). The visual stimuli was presented at different intervals after the reward consumption: **A)** 3 seconds first 15 trials, **B)** 6 seconds second 15 trials, **C)** 12 seconds third 15 trials, **D)** 24 seconds forth 15 trials. Only animals that reached the criteria of finishing minimum 50 out of 60 trials at a maximum time of 45 minutes per session were included in the analysis.

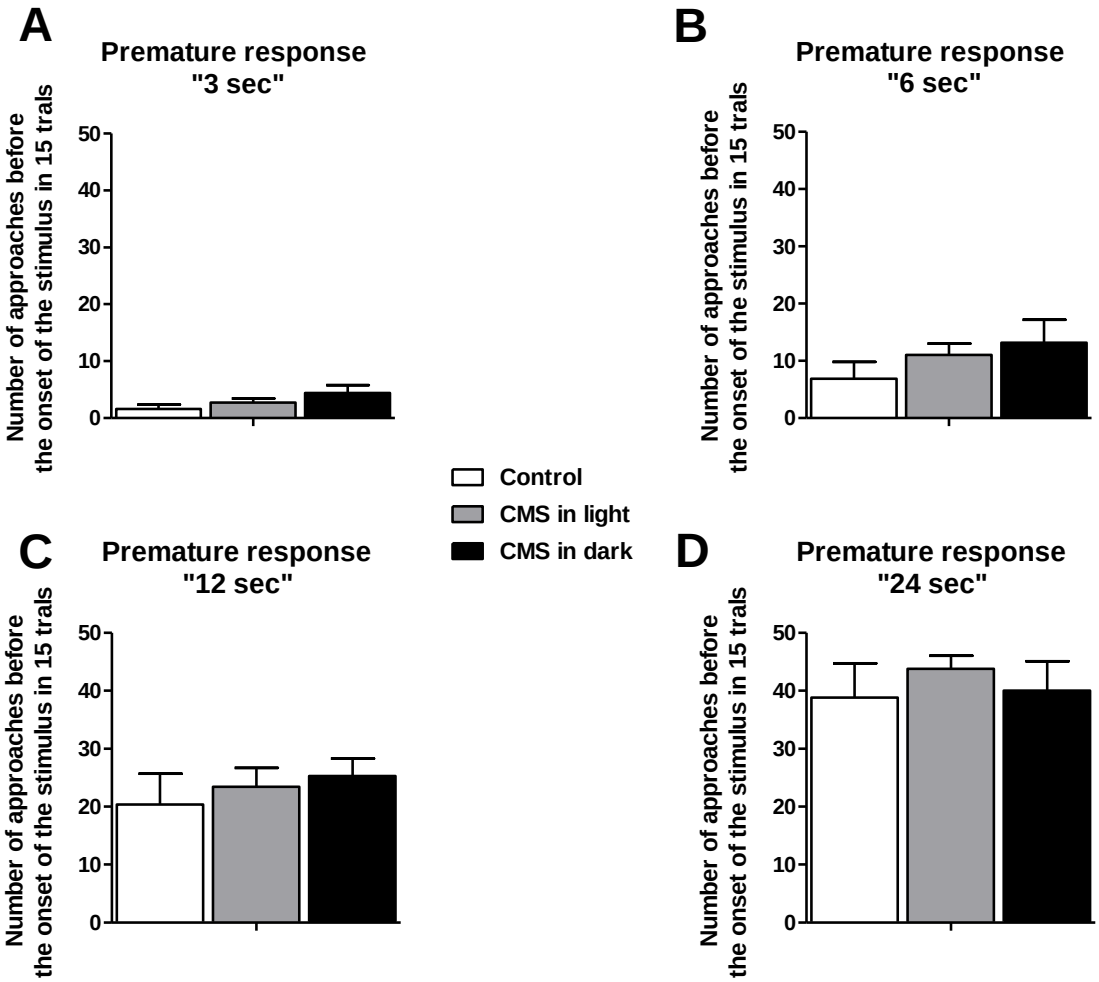

Figure A.4 Number of approaches before the onset of the stimulus in 15 trials (15trials x 4). The visual stimuli was presented at different intervals after the reward consumption: **A)** 3 seconds first 15 trials, **B)** 6 seconds second 15 trials, **C)** 12 seconds third 15 trials, **D)** 24 seconds forth 15 trials. Only animals that reached the criteria of finishing minimum 50 out of 60 trials at a maximum time of 45 minutes per session were included in the analysis.

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ANNEX II:

Day and night: diurnal phase influences the response to chronic mild stress

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Day and night: diurnal phase influences the response to chronic mild stress

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Chronic mild stress (CMS) protocols are widely used to create animal models of depression. Despite this, the inconsistencies in the reported effects may be indicative of crucial differences in methodology. Here, we considered the time of the diurnal cycle in which stressors are applied as a possible relevant temporal variable underlying the association between stress and behavior. Most laboratories test behavior during the light phase of the diurnal cycle, which corresponds to the animal's resting period. Here, rats stressed either in their resting (light phase) or active (dark phase) periods were behaviorally characterized in the light phase. When exposure to CMS occurred during the light phase of the day cycle, rats displayed signs of depressive and anxiety-related behaviors. This phenotype was not observed when CMS was applied during the dark (active) period. Interestingly, although no differences in spatial and reference memory were detected (Morris water maze) in animals in either stress period, those stressed in the light phase showed marked impairments in the probe test. These animals also showed significant dendritic atrophy in the hippocampal dentate granule neurons, with a decrease in the number of spines. Taken together, the observations reported demonstrate that the time in which stress is applied has differential effects on behavioral and neurostructural phenotypes.

Keywords: behavioral test, chronic mild stress, depression model, diurnal phase, stress

INTRODUCTION

Stressful life events predispose individuals to a number of neuropsychiatric disorders, especially depression. Depression is a devastating disease with a high rate of relapse for which there are still no effective treatments for more than 30% of the patients, despite decades of research (Kendler et al., 2001; Elizalde et al., 2010; Bartlang et al., 2012). The effects of stress depend on an interaction of multiple factors such as the quality (e.g., physical vs. psychological), intensity and chronicity of the applied stressor. Accordingly, the outcome of any particular stress paradigm can be potentially altered by very subtle procedural differences (Patchev and Patchev, 2006).

Chronic mild stress (CMS) is widely used to induce symptoms of depression in animals (Willner et al., 1992; Willner, 2005). Nonetheless, differences in the CMS protocol used by different researchers, resulting in a possible inadequacy or inappropriate use of the model, can likely be a contributor to the relatively poor rate of success in developing effective antidepressants. In agreement, the literature contains conflicting reports on the effects of CMS in terms of anhedonia, a key sign of depression (D'Aquila et al., 1994; Konkle et al., 2003; Grønli et al., 2005; Bessa et al., 2009a,b), and anxiety-like behaviors (D'Aquila et al., 1994; Gouirand and Matuszewich, 2005). These discrepancies are likely to result from methodological differences between different laboratories. An important variable that is usually not carefully described concerns the diurnal phase when CMS is applied. This is important because laboratory rodents are nocturnal and the salience of environmental stimuli, as well as the perception and response to such, may be a function of their periods of activity. In fact, when studying the effects of chronic restraint stress, Rybkin et al. (1997) and Perez-Cruz et al. (2009) concluded that the diurnal phase has an important impact on the observed behavioral phenotype. To the best of our knowledge, no study has been designed to specifically assess the effect of the diurnal phase in which stress is applied in the CMS model, which we address here.

MATERIALS AND METHODS

ANIMALS

A total of 48 male Wistar rats (Charles-River Laboratories, Barcelona, Spain), 3 months old, were used in accordance with European Union regulations (Directive 86/609/EEC) and the National Institutes of Health guidelines on animal care and experimentation. Rats were housed (2 per cage) under standard laboratory conditions (room temperature 22°C; humidity 55%; food and water *ad libitum*). Animals were exposed to normal light cycle (lights on for 12 h starting at 08:00) or inverted light cycle (lights on for 12 h starting at 20:00). The normal light cycle was automatically controlled. The inverted cycle condition was attained by covering the animals' cage with black polypropylene (light-proof boxes; 48 × 30 × 46 cm) during the facility's light phase.

The boxes were designed to assure proper ventilation and temperature maintenance similar to the standard laboratory cages. Animals in the inverted cycle condition were kept outside the black boxes in a separate room with lights on during the facilities' dark phase, and kept in inverted light condition 2 weeks before the start of the experimental procedures. Each group (normal light cycle vs. inverted light cycle) was further subdivided into 2 subgroups: control (not being disturbed besides handling) and CMS (exposed to a chronic mild stress protocol).

Animals were weighed once a week. Control rats were handled frequently during the experimental period and all groups were also handled for 5 min per day on the week before the start of the behavioral testing. Behavioral testing was performed 1 h after the start of the light phase of the diurnal cycle (animals resting period).

To avoid potentially confounding effects of different tests, these followed a specific order according to the sensitivity of each of them. As such, the following order was applied: elevated plus maze (EPM), open field (OF), forced swimming test (FST) and Morris water maze (MWM). **Figure 1** depicts the scheme of the experimental approach and the order of the behavioral tests.

CHRONIC MILD STRESS (CMS)

The CMS protocol used was a slightly modified version of an unpredictable CMS protocol (Willner, 2005). Over a period of 6 weeks, it included a battery of chronic unpredictable mild stressors, namely: confinement to a restricted space for 2 h, placement in a tilted cage (30°) for 4 h, housing on damp bedding for 8 h, 12 h food deprivation followed by exposure to inaccessible food for 1 h, water deprivation for 12 h followed by exposure to an empty bottle for 1 h. The CMS was induced either in the light phase of the diurnal cycle (CMS-Light) or in the dark phase of the diurnal cycle (CMS-Dark).

SUCROSE PREFERENCE TEST (SPT)

The SPT was performed in 3 time points throughout the CMS protocol: baseline (before the start of the CMS), in the middle (at the end of third week of the CMS) and at the end (at the end of last week of the CMS). After a 22 h food and water deprivation, animals were presented with two pre-weighed bottles containing 2% sucrose solution or tap water for 1 h. Sucrose preference was calculated according to the formula: sucrose preference = [sucrose intake/(sucrose intake + water intake)]*100. Anhedonia was defined as a reduction in sucrose solution consumption relative to baseline levels (Bekris et al., 2005).

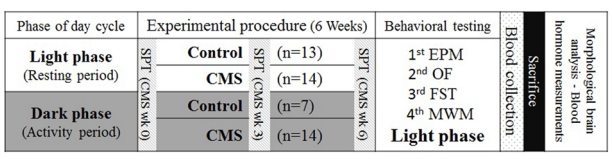

FIGURE 1 | Experimental timeline. Rats were housed in different light phases and the CMS protocol was applied to both CMS groups at the same time. Behavioral tests were performed, in the order shown (third column), immediately after the CMS protocol (6 weeks), during the daily period of the light-dark cycle. SPT, sucrose preference test (at 3 time points); EPM, elevated plus maze; OF, open field; FST, forced swimming test; MWM, Morris water maze. Morphological and blood hormone analysis were performed after the sacrifice.

FORCED SWIMMING TEST (FST)

Learned helplessness, another dimension of depressive-like behavior, was evaluated with FST. Briefly, 24 h after a pre-test session (8 min), rats were placed in cylinders filled with water (25°C) to a depth such that the animals had no solid support. The actual test lasted for 5 min and was assessed using a camera. Learned helplessness behavior was defined as an increase in the time of immobility (defined as time spent either immobile or in movements to stay afloat) (Porsolt et al., 1978). An investigator blind to the experimental details scored the video recordings.

ELEVATED PLUS MAZE (EPM)

The EPM was used to test anxiety-like behaviors. The EPM apparatus was made of a black polypropylene plus shaped platform (ENV- 560; Med Associates Inc, St Albans, VT, USA), which was elevated 72.4 cm above the floor and consisted of two opposite open (50.8 × 10.2 cm) and closed arms (50.8 × 10.2 × 40.6 cm). Rats were placed individually in the center of the maze and their ambulation was monitored online with an infrared photobeam system over a period of 5 min (MedPCIV, Med Associates Inc.). The ratio of the time spent in the open versus closed arms was used as an index of anxiety-like behavior.

OPEN FIELD (OF)

Locomotor and exploratory behaviors were investigated using the OF test. Briefly, rats were placed in the center of a brightly illuminated arena (Med Associates Inc.) and were allowed to explore it for 5 min. Exploration and the time and distances in the central and pre-defined peripheral areas were recorded online by two 16-beam infrared arrays. Total distances traveled were calculated as indicators of locomotor activity.

MORRIS WATER MAZE (MWM)

An evaluation of cognitive function was performed in spatial working and reference memory tasks and in a reverse learning task in the MWM, as described previously (Cerqueira et al., 2007a). The MWM test was conducted in a circular black tank (170 cm diameter; depth: 50 cm) filled with water (23°C, around 30 cm depth) and placed in a dimly lit room with extrinsic visual clues. The tank was divided into imaginary quadrants and a hidden platform (12 cm diameter, submerged 2 cm below the surface of the water) was placed in the center of one of them. Data were collected using a video-tracking system (Viewpoint, Champagne au Mont d'Or, France). The spatial working memory test (Morris, 1984) was assessed in 4 consecutive days (4 trials per day, maximum of 2 min per trial). Test sessions begun with rats being placed in the tank, facing the wall of the maze, at a different starting point (in one of the imaginary quadrants in each session) and finished once the platform was found or if 2 min had elapsed (thereafter the animal was gently guided to the platform). On each trial day, the position of the platform was kept constant, but it was varied on each successive day such that all four quadrants were used. The distance traveled and the time spent to reach the platform (escape latency) was evaluated.

After assessing working memory, animals were tested for spatial reference memory. For this, the platform remained in the same quadrant to ensure that the animals correctly learned the position of the platform before assessment of reversal learning (further confirmed in the probe test). All of the remaining procedures were similar to the ones described for the working memory task. For the reverse learning task, after the animals learned the position of the platform, the escape platform was positioned in a new (opposite) quadrant and rats were tested in a 4-trial paradigm, as described above. For this task, distance and time spent swimming in each quadrant were recorded. The difference between distances traveled in the quadrant containing the newly positioned platform ("new") and the quadrant that previously contained the platform ("old") was calculated as a measure of reversal performance. In the water maze paradigm, the daily trial-to-trial progression of the distance swum to reach the platform was averaged for the different platform locations, whereas in the reference memory, day-to-day progression was averaged across the 4 daily trials for the same platform location.

CORTICOSTERONE DETERMINATIONS

Blood samples, from each rat, were collected every 6 h during 24 h, by lancing the tip of the rats' tail and collecting blood drops into a microtube. Serum was obtained and stored at −80°C for later analysis. Serum corticosterone levels were determined by radioimmunoassay (MP Biochemicals, Costa Mesa, CA).

MORPHOLOGICAL ANALYSIS

Rats were perfused transcardially with 0.9% saline under deep anesthesia and the brains processed according to the protocol described by Gibb and Kolb (1998) for the Golgi analysis. Briefly, brains were immersed in Golgi-Cox solution (1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate) (Glaser and Van der Loos, 1981) immediately after perfusion and remained for 20 d. Brains were subsequently transferred to a 30% sucrose solution (3 d), before being cut by vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto cleaned, gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated through a graded series of ethanol, cleared in xylene, mounted and cover slipped.

For the dendritic analysis of hippocampal dentate granule cells, neurons were chosen based on the following criteria: (1) cells being isolated from surrounding neurons, (2) full impregnation of the neurons, (3) cells location in the dentate gyrus region of the hippocampus (DG), and (4) no morphological changes attributable to incomplete dendritic impregnation of Golgi-Cox staining. For each selected neuron, all branches of the dendritic tree were reconstructed and the spine shape and numbers were determined at ×100 magnification using a motorized microscope (Axioplan 2, Carl Zeiss, Germany), attached to a camera (DXC-390, Sony Corporation, Tokyo, Japan), and the Neurolucida software (MicroBrightField Bioscience, Magdeburg, Germany). In order to minimize selection bias, slices containing the region of interest were randomly searched and the first 10 neurons fulfilling the above criteria were selected from each brain (Cerqueira et al., 2007b). The number of different dendritic spines was estimated by counting different spine shapes, namely thin, thick, ramified, and mushroom, in segments of the dendrites of dentate granule cells. After establishing the density of spines per category, their total number was calculated for each neuron [(number of spines/dendritic length)*total dendritic length].

STATISTICAL ANALYSIS

Homogeneity of the data was assessed before statistical analysis was performed. For the data on body weight changes, spatial reference and working memory in MWM, and Sholl analysis of dendrites in the DG, repeated measures ANOVA was used; different time points in corticosterone analysis were evaluated by *t*-test. One-Way ANOVA analysis was performed for EPM, OF, FST, SPT, reverse learning and probe task in the MWM, dendrite length and spine numbers of DG neurons; Bonferroni *post-hoc* analyses were used for comparing differences between the experimental groups. Results are expressed as means ± s.e.m. and statistical significance was accepted for $P < 0.05$. Behavioral and morphological analyses were done in two independent sets of animals. Since no statistical differences were found between these two groups (data not shown), the two sets of animals were grouped; therefore, figures and statistical analysis result from the combination of both sets of animals.

RESULTS

Comparison of the two control groups (normal and inverted light) did not reveal significant differences (**Table 1**). Therefore, with exception for the analysis of CORT determinations, the subsequent statistical analyses were performed by merging control data in a single group (CON), for clarity of comparison.

All groups of animals showed gains in body weight over the course of the experiment (**Figure 2**). A significant difference in body weight gain was observed between groups [$F_{(2, 45)} = 4.99$, $P = 0.011$]. Animals who were exposed to CMS in the light phase of the diurnal cycle (CMS-Light) gained remarkably less weight than the CON group during the stress exposure period ($P = 0.015$). Interestingly, this difference was not observed in animals exposed to stressors in their dark phase of the diurnal cycle (CMS-Dark) ($P = 0.98$); consequently, the CMS-Light and CMS-dark groups also showed significant differences ($P = 0.042$).

Anhedonia was evaluated by the sucrose preference changes relative to individual's baseline preference. The pattern indicated a reduction in sucrose solution consumption after exposure to CMS [$F_{(2, 44)} = 4.50$, $P = 0.017$]. However, this decrease was only significant in the CMS-Light animals when compared to CON (CMS-Light/CON: $P = 0.014$; CMS-Dark/CON: $P = 0.358$; CMS-Light/CMS-Dark: 0.606) (**Figure 3A**). In accordance, immobility time in the FST was remarkably increased in the CMS-Light group [$F_{(2, 45)} = 4.49$, $P = 0.017$; CMS-Light: $P = 0.018$ when compared to CON] indicating the development of depressive-like behavior. Such phenotype, however, was not observed in CMS-Dark animals (CMS-Dark/CON = 0.99; CMS-Dark/CMS-Light; 0.089) (**Figure 3B**).

Anxiety assessment in the EPM test indicated a CMS effect on the time spent in open arms in different groups [$F_{(2, 44)} = 3.30$,

Table 1 | Statistical analysis of two control (normal and inverted light) groups together.

Measurement	Statistical analysis
Normalized body weight changes	$ANOVA_{rm}$, $F_{(1, 18)} = 0.07$, $P = 0.80$
Sucrose preference test (% sucrose preference)	$ANOVA_{rm}$, $F_{(1, 17)} = 0.18$, $P = 0.68$
Elevated plus maze (% time spent in open arm)	$t_{(14.84)} = -1.2$, $P = 0.25$
Open field (% distance in center)	$t_{(12.32)} = -0.32$, $P = 0.75$
Open field (% total distance)	$t_{(10.3)} = -0.51$, $P = 0.62$
Forced swim test Immobility time	$t_{(10.58)} = -1.72$, $P = 0.11$
Morris water maze (MWM) (Spatial memory)	$ANOVA_{rm}$, $F_{(1, 18)} = 3.82$, $P = 0.07$
MWM (Reference memory)	$ANOVA_{rm}$, $F_{(1, 18)} = 3.96$, $P = 0.06$
MWM—reversal memory task (Distance traveled in new quadrant)	$t_{(8.8)} = 0.6$, $P = 0.56$
MWM—reversal memory task (Distance traveled in old quadrant)	$t_{(7.4)} = -0.29$, $P = 0.78$
MWM—probe test (Distance traveled in target quadrant)	$t_{(1.7)} = 15.6$, $P = 0.11$
Total dendritic length (DG)	$t_{(16.5)} = -1.04$, $P = 0.312$
Sholl analysis (DG)	$ANOVA_{em}$, $F_{(1, 20)} = 0.57$, $P = 0.46$
Total number of thin-type spine (DG)	$t_{(11)} = -0.62$, $P = 0.547$
Total number of mushroom-type spine (DG)	$t_{(5.66)} = -1.283$, $P = 0.25$
Total number of thick-type spine (DG)	$t_{(7.64)} = 0.894$, $P = 0.398$
Total number of ramified-type spine (DG)	$t_{(11)} = -0.29$, $P = 0.777$
Total number of spines (DG)	$t_{(11)} = -0.05$, $P = 0.961$

Repeated measurements and t-test analysis indicate that there are no significant differences between the two control groups in any of the measurements.

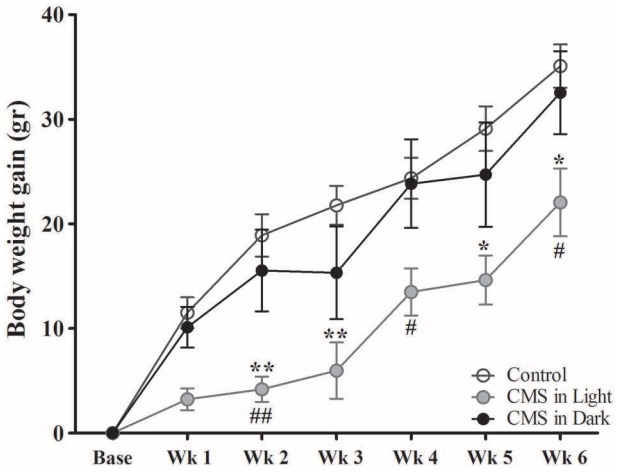

FIGURE 2 | Body weight gain during the CMS protocol. Controls, $n = 20$, each CMS group, $n = 14$; $^{*,\#}P < 0.05$. $^{**,\#\#}P < 0.01$. Data presented as mean ± s.e.m. * indicates a significant difference between the CMS-Light and the CON group and # show a significant difference between the CMS-Light and CMS-Dark. There were no significant differences between the CMS-Dark and the CON group.

$P = 0.046$]. Nevertheless, only the CMS-Light group spent significantly less time in the open arms compared to CON animals ($P = 0.043$). The CMS-Dark rats did not differ in this parameter from CON ($p = 0.99$) or CMS-Light ($p = 0.36$) (**Figure 3C**). In the OF test, no differences were observed between group in distance traveled in the center of the arena [$F_{(2, 40)} = 1.07$, $P = 0.353$] (**Figure 3D**) and in total distances traveled [$F_{(2.39)} = 2.77$, $P = 0.075$] (**Figure 3E**). The former parameter is a measure of anxiety-like behavior, whereas the latter is an index of locomotor behavior.

The learning curve in spatial working (**Figure 4A**) and reference (**Figure 4B**) memory tasks in the MWM did not show differences between groups [$F_{(2, 45)} = 1.26$, $P = 0.294$; $F_{(2, 43)} = 1.11$, $P = 0.338$ respectively]. Similarly, no differences were observed in the reversal learning task performance (a test for behavioral flexibility measurement) [distance swum in the "new" quadrant: $F_{(2, 44)} = 2.04$, $P = 0.142$; distance swum in the "old" quadrant: $F_{(2, 43)} = 1.82$, $P = 0.175$] (**Figure 4C**). The significant difference in the analysis of the probe test was due to considerably decrease in the distance swum in the target quadrant in CMS-Light animals [$F_{(2, 41)} = 8.23$, $P = 0.001$; $P = 0.043$] when compared to the CON group; this difference was also observed between CMS-Light and the CMS-Dark groups ($P = 0.001$). The CMS-Dark group however, did not differ from the CON group ($P = 0.246$) (**Figure 4D**).

Indicating for different stress effects regarding different phases of the diurnal cycle, only the CMS-Light group revealed disrupted circadian rhythm on the corticosterone profile (when all groups were compared together). These group differences were found mostly in the animals' light phase, at zeitgeber time (Zt) 0 [$t_{(24)} = -2.89$, $P = 0.008$] and Zt 6 [$t_{(25)} = 3.22$, $P = 0.004$] which correspond to the animal's resting period (**Figure 5**). Morphological analysis in the DG revealed a decreased dendrite length in granule neurons of animals exposed to CMS [$F_{(2, 45)} = 3.8$, $P = 0.03$]; this difference again was only significant in the CMS-Light group (CMS-Light/CON: $P = 0.026$; CMS-Dark/CON: 0.99; CMS-Light/CMS-Dark: $P = 0.3$) (**Figure 6A**). This observation was confirmed by the Sholl and spine density analysis of the same neurons; with the Sholl data [$F_{(2, 45)} = 4.36$, $P = 0.02$] indicating a decrease in the number of intersections with significant group difference between the CMS-Light and other groups (CMS-Light/CON: $P = 0.025$; CMS-Dark/CON: 0.99; CMS-Light/CMS-Dark: $P = 0.58$) (**Figure 6B**).

Exposure to CMS led to a reduction in the overall spine number [$F_{(2, 28)} = 5.59$, $P = 0.009$] in the hippocampal dentate gyrus (**Figure 6C**). This decrease was observed only in the animals that were stressed in their resting period (CMS-Light/CON: $P = 0.01$; CMS-Dark/CON: 0.99; CMS-Light/CMS-Dark: $P = 0.57$). More specifically, a statistically significant decrease was found in two spine types, namely: mushroom spines [$F_{(2, 26)} = 5.47$,

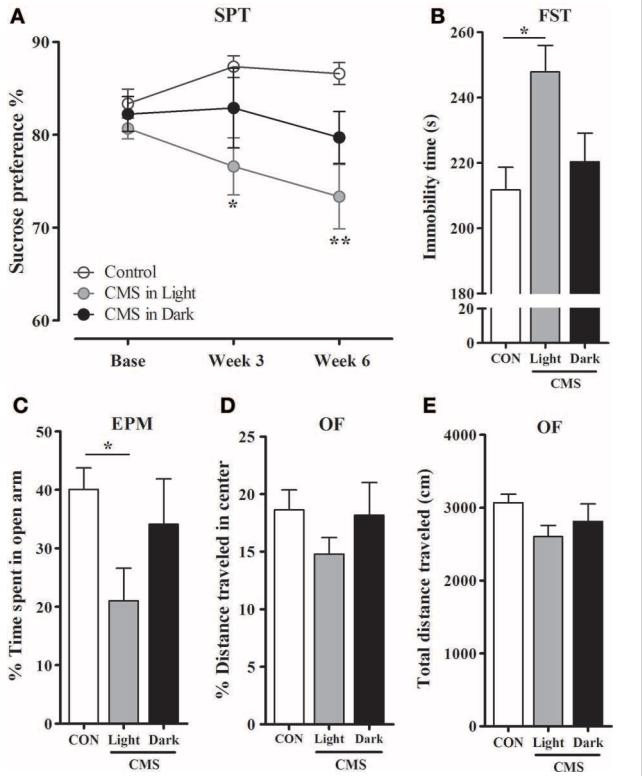

FIGURE 3 | Emotional behavior in rats exposed to CMS in different diurnal phases. (A) Preference changes of sucrose consumption in the Sucrose preference test (SPT). * indicates a significant difference between the CMS-Light and CON. There were no significant differences between the CMS-Dark and the two other groups. **(B)** Forced swimming test (FST). Immobility time during the test period. **(C)** Elevated plus maze (EPM). Percentage of time spent in the open arms **(D–E)** Open field test (OF). **(D)** Percentage of the distance traveled in the center and **(E)** Total distance traveled in the OF apparatus. Controls, $n = 20$, each CMS group, $n = 14$; $^{*}P < 0.05$; $^{**}P < 0.01$. Data presented as mean ± s.e.m.

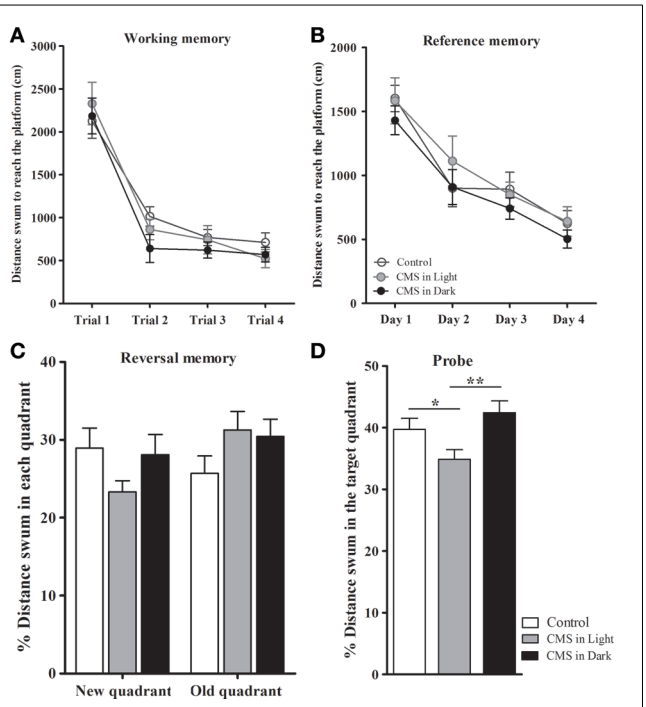

FIGURE 4 | Impact of CMS during different phases of the day light-dark cycle on spatial working, reference and reversal memory. (A) Average distance swum during the spatial working memory task. **(B)** Average distance swum during the reference memory task. **(C)** Percentage of distance swum in the new/old quadrant during the reversal memory task. **(D)** Percentage of distance swum in the target quadrant during the probe test. Controls, $n = 20$, each CMS group, $n = 14$; $^{*}P < 0.05$. $^{**}P < 0.01$. Data presented as mean ± s.e.m.

$P = 0.01$; CMS-Light/CON: $P = 0.01$; CMS-Dark/CON: 0.99; CMS-Light/CMS-Dark: $P = 0.69$], and thick spines [$F_{(2,\ 28)} = 4.55$, $P = 0.02$; CMS-Light/CON: $P = 0.017$; CMS-Dark/CON: 0.44; CMS-Light/CMS-Dark: $P = 0.53$]. This difference was not observed in thin [$F_{(2,\ 28)} = 2.85$, $P = 0.075$] and ramified spines [$F_{(2,\ 28)} = 1.2$, $P = 0.317$] (**Figure 6D**).

DISCUSSION

This study shows that the effect of exposure to CMS depends on the phase of the diurnal cycle in which the CMS procedure is applied. Depressive and anxiety-related behaviors and memory impairments only occur when rats are exposed to CMS during their daily period of rest (light phase). Similarly, CMS during the inactive, but not active, day period leads to body weight loss, altered corticosterone secretory profiles, and reduced dendritic arborization and number of mature (thick, ramified, and mushroom) spines in the granule cells of the hippocampus. These findings clearly show that rats can cope with CMS during their active period. Results are relevant when considering that most studies reported in the literature use animal models in which stress is induced during the resting phase. In addition, these observations should be taken into consideration when studies on animal models of CMS serve as basis to infer on the consequences of human exposure to stress.

Delayed body weight gain (in younger subjects) and weight loss are well known consequences of chronic stress (Barr and Phillips, 1998; Bielajew et al., 2002; Konkle et al., 2003). In the present study, CMS during the daily resting (light) period caused significant loss of body mass; the same treatment during the dark phase of the diurnal cycle resulted in only minor fluctuations in body mass. Results are in accordance with those reported by Grønli et al. (2005), which is one of the few laboratories, to our best knowledge, that has performed CMS in the dark phase of the light cycle. Previous studies have shown that disruption during the resting phase interferes with the pattern of food ingestion and metabolism (Nagano et al., 2003; Salgado-delgado et al., 2008, 2011), suggesting disruption of the circadian regulation of feeding and metabolism. Interestingly, glucocorticoid secretion normally follows a tight circadian rhythm, with peak secretion occurring just at the onset of the daily period of darkness when rodents show high levels of locomotor activity and feeding. Here we found the rhythmic secretion of corticosterone to be markedly altered in rats that had experienced CMS during their inactivity period (light phase). Specifically, the corticosterone profile

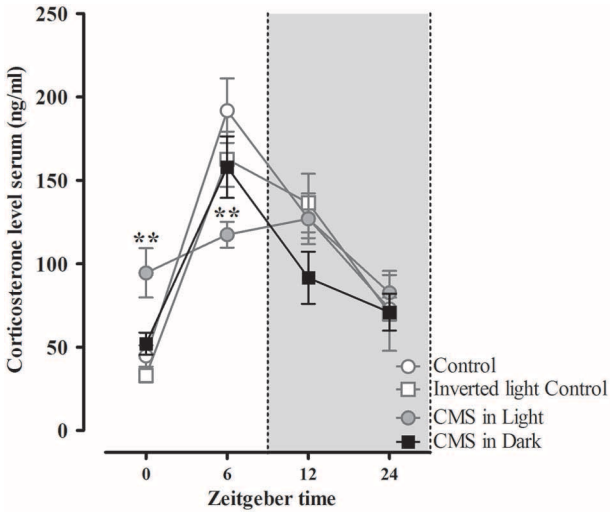

FIGURE 5 | Diurnal rhythm of corticosterone secretion in rats exposed to CMS in different phases of the day light-dark cycle. Serum corticosterone levels during 24 h post CMS, measured every 6 h. Controls, $n = 20$, each CMS group, $n = 14$; $^{**}P < 0.01$. Data presented as mean ± s.e.m. ** indicate significant differences between the CMS-Light and the CON group.

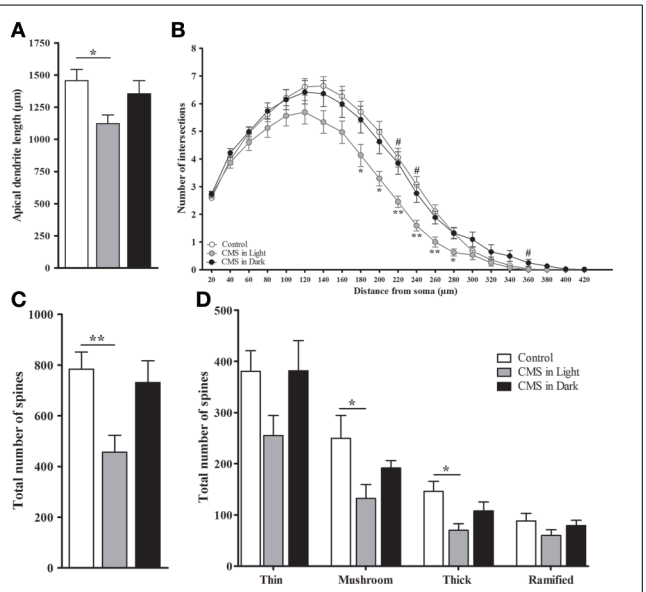

FIGURE 6 | Morphometric analysis of dendritic arborizations in the hippocampal dentate gyrus. (A) Total dendritic length. **(B)** Sholl analysis-derived distribution of dendrites. **(C)** Total number of spines. **(D)** Total number of different types of spines. Controls, $n = 20$, each CMS group, $n = 14$; $^{*}P < 0.05$. $^{**}P < 0.01$. $^{\#}P < 0.05$. Data presented as mean ± s.e.m. * and ** indicate significant differences between the CMS-Light and the CON group. # indicates a significant difference between the CMS-Light and the CMS-Dark group.

of these animals showed a sluggish rise as the nocturnal period approached, and the zenith of secretion was shifted to the middle of the dark period of the day cycle. At the same time, these animals showed higher blood levels of corticosterone at the beginning of the daily period of light. In other words, CMS applied during the animals' inactivity period resulted in a blunted and phase-shifted corticosterone rhythm. These results largely concur with those previously reported (Bielajew et al., 2002; Konkle et al., 2003; Ushijima et al., 2006). Interestingly, similar disruption of the daily glucocorticoid rhythm is also seen in a large sub-group of patients suffering from major depression (Jarcho et al., 2013). It has been shown that the inability to mount an appropriate response to stress makes individuals more vulnerable to stressors (Zimmermann and Critchlow, 1967); therefore, it is likely that this disrupted profile is critical for explaining the deleterious effects of stress during the resting phase of the animals' diurnal cycle. Indeed, there is evidence that the lack of synchrony of the internal clock, in association to altered glucocorticoid levels, might play a role in the development of emotional disturbances, namely depression (Salgado-delgado et al., 2011), a finding that we observed exclusively in animals exposed to CMS during the light phase of the day cycle. However, we should consider that the present study has only assessed behavior during the resting phase of rodents. Future studies should evaluate the impact of the time of testing in the behavioral phenotype of stress rodents.

Learned helplessness and anhedonia, two characteristics of depressive illness, can be induced by CMS in animals (D'Aquila et al., 1994; Elizalde et al., 2008; Bessa et al., 2009a,b). These behaviors can be assessed in the SPT and FST, respectively; however, these have not always been consistently reproduced (Konkle et al., 2003). The time of the day may contribute to such discrepancies, particularly as the present study showed that only animals exposed to CMS in their resting period displayed signs of depressive-like behavior. A previous study (based on chronic restraint stress) also noted the importance of the diurnal phase in which the stress is applied in determining responses in the SPT and FST (Huynh et al., 2011), which is in accordance with the present findings. We used the OF test to address measures of anxiety. Grønli et al. (2005), who performed CMS exposure in dark phases, failed to find signs of increased anxiety, similar to our current observations; yet, on this parameter the study of restraint stress in rats observed the opposite effect (Huynh et al., 2011).

The effects of CMS on learning and memory in rodents are controversial. For example, while CMS-induced impairments in learning have been reported (Song et al., 2006; Elizalde et al., 2008), we failed to observe deficits in spatial reference learning and memory after this treatment (Bessa et al., 2009b; present study). On the other hand, as reported herein, significant cognitive impairments were detectable by the probe test in rats exposed to CMS solely during the daily period of light.

Finding anatomical correlates of behavioral changes may help to understand the mechanisms underlying the response to stress. Bessa et al. (2009a) showed that stress induces dendritic atrophy (length, branch number and spine numbers) in hippocampal granule neurons, an area implicated in spatial learning and memory, as we also show here in animals exposed to CMS during the daily period of inactivity (light). This reduction in the dendritic arbor and in the number of spines, particularly mature spines, is likely to translate into synaptic signaling decrease (Kennedy et al.,

2005), and in impairments in hippocampal-based learning and cognition abilities (Bliss and Collingridge, 1993). Moreover, since the hippocampus exerts inhibitory control over the HPA axis, we suggest that these structural changes might also translate into reduced inhibitory input to the hypothalamus, thus contributing to the marked CMS-induced disruption of the corticosterone secretory profile.

The experiments reported here help clarify discrepancies in the literature regarding the robustness of the CMS paradigm for the induction of depressive- and anxiety-like behaviors in rats. Briefly, our results show that CMS is only effective when applied during the daily period of the diurnal cycle when animals are inactive, primarily sleeping. CMS administered during the daily period of activity does not trigger depressive- or anxiety-like behaviors and may lead to the false presumption that the method is invalid or that animals are resilient to the deleterious effects of stress.

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