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Molecular mechanisms of the synaptic and cognitive effects of ghrelin

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor João Peça (Centro de Neurociências e Biologia Celular, Instituto de Investigação Interdisciplinar, Universidade de Coimbra) e da Professora Doutora Ana Luísa Carvalho (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra)

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iv. ABBREVIATIONS

2-AG	2 arachidonoylglycerol
ACTH	adrenocoticotropin hormone
AKAP97	A kinase anchoring protein 97
AgRP	agouti gene-related peptide
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors
AMPK	AMP-activated protein kinase
AP	area postrema
AP-2	activating protein 2
AP5	D-(-)-2-amino-5-phosphonopentanoic acid
ARC	arcuate nucleus
BBB	blood-brain barrier
CA1	cornu ammonis area 1
Calcrl	calcitonin receptor-like
CAMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CART	cocaine and amphetamine-regulated transcript
CasR	calcium-sensing receptor
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CKK	cholecystokinin
CREB	camp response element-binding protein
CRH	corticotrophin-releasing hormone
CSDS	chronic social defeat stress
D1R	dopamine receptor 1
DAG	diacylglycerol
DLB	dark-light box
DMV	dorsal motor nucleus of the vagus
DRN	dorsal raphe nucleus
EphB	ephrin-type B receptor
EPM	elevated-plus maze
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
FFAR2	free fatty acid receptor
GH	growth hormone

GHS	growth hormone secretagogue
GHS-R1a	growth hormone secretagogue receptor 1a
GHS-R1b	growth hormone secretagogue receptor 1b
GHSR	growth hormone secretagogue receptors
GIP	gastric inhibitory peptide
GLP-1	glucagon-like peptide 1
GluN1	glun1 subunit of nmdar receptor
GOAT	ghrelin-O-acyltransferase
GPCR	G-protein coupled receptors
GPR39	G-protein coupled receptor 39
GPR81	G-protein coupled receptor 81
GRIP	glutamate receptor interacting protein
HEK	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFS	high frequency stimulation
HPA	hypothalamic-pituitary-adrenal axis
ICV	intracerebroventricular
IGF-I	insulin-like growth factor-1
iGluR	ionitropic glutamate receptors
IP	intraperitoneal
IP3	inositol 1,4,5-triphosphate
IPSP	inhibitory postsynaptic potential
LASGB	laparoscopic adjustable silicone gastric banding
LH	latereal hypothalamus
LRYGBP	laparoscopic roux-en-y gastric bypass
LTP	long-term potentiation
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
MC4R	melanocortin 4 receptor
mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
Nacc	nucleus accumbens
NF-IL6	nulcear factor for il-6 expression
NF-kB	nuclear factor kappa b
NMDAR	n-methyl-d-aspartate-type receptor
NMU-R1	neuromedi-u receptor 1

NOS	nitric oxide synthase
NPY	neuropeptide y
NTS	nucleus tractus solitarius
NTS-R	neurotensin receptor
OF	open-field
PC 1/3	proprotein convertase 1
PDZ	postsynaptic density 95/disc large/zonula occludens 1
PFH	perifornical hypothalamus
PLC	phospholipase C
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
RYGBP	roux-en-y gastric bypass
SAMP8	senescence-accelerated prone mouse 8
SHANK	SH3 and multiple ankyrin repeat domains protein family
SRE	serum response factor
SSRI	selective serotonin reuptake inhibitor
SSTR1	somatostatin receptor 1
SV40	simian vacuolating virus 40
SynGAP	synaptic ras gtpase-activating protein
TARP	trasmembrane ampa receptor regulating protein
TM	trasmembrane
VM	ventromedial
VTA	ventrotegmental area

v. ABSTRACT

Ghrelin was discovered in 1999 by Kojima and co-workers as a ligand for the growth hormone secretagogue receptor (GHSR) (Kojima and Kangawa, 2005). It was subsequently discovered that ghrelin is secreted by the stomach during periods of fasting and is the only known peripheral orexigenic hormone that acts on the hypothalamic circuits regulating food intake and energy balance. Additionally, it was also found that circulating levels of ghrelin oscillate throughout the day (Cummings et al., 2001a). In 2002, work by Carlini and co-workers unraveled memory-enhancing properties of ghrelin (Carlini et al., 2002a). Today it is well established that acts on diverse brain regions it mediating its memory-enhancing effects through several mechanisms. The orexigenic and memory enhancing effects of ghrelin fit within an evolutionary and ethological role for this hormone in the ability of animals to remember the location and condition of food sources (Moran and Gao, 2006). Recent work by Carvalho and colleagues elucidated a novel molecular mechanism through which ghrelin can exert its memory enhancing effects (Ribeiro et al., 2014). Specifically, it was shown that activating GHSR leads to the incorporation of GluA1 in hippocampal synapses and facilitates NMDAR-dependent long-term potentiation.

This master thesis aims at continuing the elucidation of the mechanisms behind ghrelin's effect on memory. Taking into account the oscillating levels of ghrelin throughout the day, we decided to investigate the molecular effects of the abrupt decrease in ghrelin levels that naturally occur upon the act of feeding. We observed that this abrupt decrease of ghrelin levels robustly decreases the levels of hippocampal synaptic GluA1 without affecting the levels of NMDA receptor GluN1 and the synaptic scaffolding protein PSD-95. This observation constitutes a previously undescribed phenomenon that can be integrated within the physiological framework of memory enhancement by ghrelin. We also aimed at clarifying whether an increase in ghrelin levels produces anxiolytic or anxiogenic effects *in vivo*. We observed that ghrelin mainly decreases anxiety, but depending on the interval between ghrelin administration and behavioral testing and the behavioral paradigm used, the effects may be confounded by a superposed decrease on motor activity. The knowledge of how ghrelin modulates anxiety is essential for the interpretation of results of *in vivo* tests on memory.

The results of this thesis work advance the knowledge of the molecular mechanism of ghrelin modulation of memory. More in-depth experiments can be done on the basis of the knowledge here presented.

Keywords: Ghrelin system, glutamatergic synapse, memory, anxiety

vi. RESUMO

A hormona grelina foi descoberta em 1999 por Kojima et al., atuando como um ligando para o receptor secretagogo de hormona de crescimento (GHSR) (Kojima and Kangawa, 2005). Foi posteriormente descoberto que a grelina é a única hormona orexigénica periférica que atua sobre circuitos hipotalâmicos de regulação do consumo de alimentos e balanço energético. Os níveis plasmáticos de grelina oscilam ao longo do dia (Cummings et al., 2001a). Em 2002, Carlini et al. descreveram pela primeira vez a capacidade da grelina de melhorar a memória (Carlini et al., 2002a). Atualmente sabe-se que a grelina atua sobre diversas regiões do cérebro, mediando melhoria cognitivas através de diversos mecanismos moleculares já descritos. Estas melhorias cognitivas por parte da grelina têm relevância evolutiva sendo vantajoso para um organismo que a capacidade de memorizar a localização e condição de fontes de alimento seja melhorada por moléculas envolvidas no balanço energético e fome (Moran and Gao, 2006). Trabalho recente no laboratória de Ana Luísa Carvalho elucidou um novo mecanismo molecular através do qual a grelina poderá exercer os seus efeitos de melhoria cognitiva (Ribeiro et al., 2014). Foi demonstrado que a activação do recetor de grelina leva à incorporação sinática de GluA1 no hipocampo e facilita a potenciação sinática a longo-prazo dependente de receptores NMDA.

O trabalho desta tese tem como objetivo continuar a elucidação dos efeitos da grelina na memória. Tendo em conta a oscilação dos níveis de grelina ao longo do dia, foi explorado o efeito de reduções abruptas dos níveis de grelina, que nos animais corresponde ao período imediatamente após a refeição. Foi demonstrado que a diminuição abrupta dos níveis de grelina leva a uma diminuição robusta dos níveis de receptores GluA1 sináticos no hipocampo, sem que os níveis de recetores NMDA GluN1 ou PSD-95 fossem afetados. Estes resultados constituem uma observação inédita que poderá ser integrada em um mecanismo fisiológico de melhoria cognitiva mediado pelo grelina. Tivemos também como objetivo clarificar o efeito da grelina na ansiedade, que constitui atualmente uma questão aberta na literatura. Foi demonstrado que a grelina em geral diminui a ansiedade, embora dependendo do período de tempo esperado entre a administração de grelina e a realização de testes comportamentais e o teste comportamental usado, este efeito possa ser obscurecido por uma diminuição na atividade motora. A caracterização do efeito da grelina na ansiedade é essencial para a interpretação de resultados de testes de memória *in vivo*.

Os resultados desta tese avançam o conhecimento dos mecanismo moleculares de modulação da memória pela grelina. Experiências mais aprofundadas podem ser realizadas com base neste conhecimento.

Palavras-chave: Grelina, sinapse glutamatérgica, memória, ansiedade

Chapter 1 – Introduction and Objectives

1 INTRODUCTION

1.1 GHRELIN SYSTEM

The molecular components of the ghrelin system consist mainly of one receptor, the growth hormone secretagogue receptor type 1a (GHSR1a), ghrelin, the endogenous agonist of this receptor, the obstatin hormone, which is derived from the same precursor as ghrelin, and the enzyme ghrelin O-acyltransferase (GOAT), which catalyzes the transfer of an acyl group to ghrelin, converting it to its active form (Gueorguiev and Korbonits, 2013). There are several other receptors directly related or with a high degree of homology to GHSR1a. To date no other receptor has been identified as playing any described function within the ghrelin signaling system. Nevertheless, there are several synthetic ligands for GHSR1a and there is strong evidence suggesting that ghrelin may bind additional receptors (Caminos et al., 2005; De Vriese et al., 2005; Iglesias et al., 2004). Anatomically this system comprises several sources of ghrelin hormone, which is mainly produced in the stomach (Ariyasu et al., 2001) but also the duodenum, jejunum, ileum, colon, lungs (Date et al., 2000b; Hosoda et al., 2000a; Sakata et al., 2002a), gonads, pancreatic islets, placenta (Dezaki and Yada, 2012; Gaytan et al., 2003; Gualillo et al., 2001; Hattori et al., 2001a; Mori et al., 2000; Tena-Sempere et al., 2002; Volante et al., 2002) and the brain (Lu et al., 2002); and several tissues expressing the GHSR1a: mainly the brain (hypothalamus and pituitary, hippocampus, substantia nigra, ventral tegmental area, and raphe nuclei) and also on the heart, lung, liver, kidney, pancreas, stomach, small and large intestines, adipose tissue and immune cells (Gnanapavan et al., 2002; Guan et al., 1997b; Hattori et al., 2001b; Kojima et al., 2001). The ghrelin system key physiological role appears to be its involvement in the regulation of appetite and food intake, through the actions of ghrelin on the hypothalamic neuronal signalling. Nevertheless, ghrelin signaling has also been implicated in the regulation of gastric and cardiac function, nutrient metabolism and pancreatic function, bone formation, modulation of inflammation, reproductive functions; and regulation of learning and memory, reward, motivation, and mood through the actions of ghrelin on extra-hypothalamic brain regions (Andrews, 2011; Delporte, 2013). The GOAT enzyme also plays an important regulatory role on the ghrelin system: its activity depends on the availability of dietary lipids, which serve as its substrates, and its expression decreases with long-term starvation (Smith and Thorner, 2012). Obstatin is produced in the gastric mucosa, and other regions of the digestive tract, and the mammary glands in humans (Gronberg et al., 2008). The proposed receptors for obstatin are GPR39 and GLP-1, mediating its functions of decreasing food intake, inhibiting gastric motility and promoting adipogenesis (Zhang et al., 2013).

For the purpose of this thesis, I will begin by reviewing the current knowledge regarding the ghrelin hormone and the ghrelin receptor. I will then briefly cover the physiology of the ghrelin system, focusing on the regulation of behavior and cognition, and finally give an overview on the molecular and cellular mechanisms thought to be responsible for hippocampal-dependent memory formation. This overview of

the literature aims at clarifying the background leading up to our experiments and the milieu surrounding our hypothesis on ghrelin regulation of hippocampal-dependent memory formation.

1.2 Ghrelin System Molecular Biology

1.2.1 The Ghrelin hormone

Ghrelin is a peptide hormone, first identified as the endogenous ligand of the growth hormone secretagogue receptor type 1 (GHS-R1a or ghrelin receptor) (Kojima et al., 1999). This discovery took advantage of reverse pharmacology methods using the previously characterized GHS-R1a as bait. Ghrelin was found to be composed of 28 amino acids and the unusual post-translational modification of N-terminal Ser^3 *n*-octanoylation, which is known to be essential for its activity (Sato et al., 2012).

Ghrelin is part of the ghrelin-motilin peptide family. Motilin and ghrelin percursors have almost 50% similarity in their amino acid sequences, and motilin receptor is the closest relative of the ghrelin receptor, with 52% of identic amino acid sequence (Feighner, 1999; Tomasetto* et al., 2000). These two peptides play similar roles on the increase of stomach motility (Ohno et al., 2010), increase of gastric acid secretion (Masuda et al., 2000), increase in food intake and growth hormone secretion (Folwaczny et al., 2001). The rat ghrelin precursor and mouse motilin-related peptide have a 95% identity between them, although the acyl group post-translational addition is not observed (Coulie and Miller, 2001; Del Rincon et al., 2001). It is noteworthy that to date no cross-reactivity between the ligands have been reported, which may be explained by the considerable difference of the pharmacophore of the peptides (Peeters, 2005).

The gene for human ghrelin is localized on the short arm of the chromosome 3 (3p25-26). This gene contains six exons, two of them noncoding, and 4 introns. The first short exon has only 20 bp and encodes part of the 5'-unstranslated region (Figure 1). The functional ghrelin peptide of 28 amino acids is coded in exons 1 and 2. The transcript gives rise to the ghrelin percursor, prepro-ghrelin, of 117 aa. This percursor is composed of a 23 aa signal peptide and proghrelin 94 aa (Korbonits et al., 2004; Seim et al., 2007). Proghrelin contains the 28 aa ghrelin peptide and a 66 aa carboxyterminal peptide called C-ghrelin. The codon Gln14 (CAG) on exon 2 is used as an alternative splice site in mouse and rat genes, generating two different ghrelin mRNA: one encoding the complete ghrelin percursor, prepro-ghrelin, and another encoding a des-Gln14-ghrelin percursor which will give rise to an identical peptide to ghrelin expect for the absence of Gln14 (Hosoda et al., 2000b). Apart from the identification of this splice variant, only two published studies looked at its function, determining that it increases papillary muscle tension (Bedendi et al., 2003) and increases gastric emptying (Dornonville de la Cour et al., 2004) in a similar manner as ghrelin. Another splice variant is expressed in mouse testis, known as the ghrelin gene-derived transcript. It has a 68-bp 5'-unique sequence and includes the exons 4 and 5 of mouse ghrelin gene. Because it does not encode the ghrelin sequence, its function is not clear (Kojima

and Kangawa, 2005). Antisense transcripts, transcribed from the antisense strand, have been identified (Seim et al., 2007). These may produce noncoding RNAs involved in gene regulation. It was shown that the 66 amino acid tail of pro-ghrelin can either circulate as a full-length peptide or be processed to smaller peptides, mainly obestatin. C-ghrelin has been shown to circulate in the plasma at higher levels than ghrelin and to be elevated in heart failure, suggesting that it may be bioactive (Soares and Leite-Moreira, 2008). The already mentioned obestatin is also coded from the ghrelin/obestatin gene (Zhang et al., 2005).

Regulating elements can be found on the 5'-flanking region human ghrelin gene, such as, a TATA box-like sequence, putative binding sites for several transcription factors, namely AP2, basic helix-loop-helix (bHLH), PEA-3, Myb, NF-IL6, hepatocyte nuclear factor-5, and NF-κB, and half-sites for estrogen and glucocorticoid response elements (Kojima and Kangawa, 2005). Glucagon and its second messenger cAMP stimulates ghrelin promoter activity in ECC10 cells, a human stomach-derived cell line (Kishimoto et al., 2003).

The amino acid sequence of ghrelin is well conserved. In the precursor, the 28 amino acid active ghrelin is followed by a signal peptide, and the cleavage site for this peptide is the same in all mammalian coded forms of ghrelin (Kojima and Kangawa, 2005).

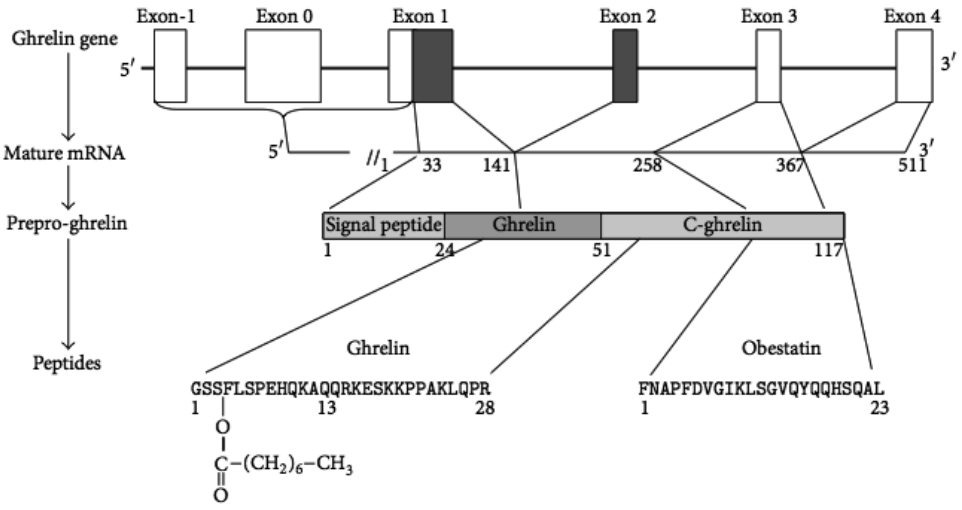


Figure 1 - From human ghrelin gene to the active peptide. The ghrelin gene has six exons, the first encoding the 5'-unstranlated region. This mRNA is translated into a 117-amino acid ghrelin percursor (prepro-ghrelin). Protease cleavage and acyl-modification of the ghrelin percursor result in the production of a 28 amino-acid-long active acyl-modified ghrelin peptide. (Adapted from **(**Delporte, 2013**)).**

During post-translational processing, prepro-ghrelin is first cleaved by a putative signal peptidase producing a 23 amino acids signal peptide from its N-terminal and pro-ghrelin, with 94 amino acids (Garg, 2007). Pro-ghrelin is further cleaved at Arg23 by prohormone convertase 1/3 (PC 1/3) (Zhu et al.,

2006), generating ghrelin 1-28, and at Pro50 by caboxypeptidase-like B enzyme, generating ghrelin 1-27 (Hosoda et al., 2003). The serine-3 of ghrelin is modified by acylation with an octanoyl group, an essential modification for the hormone to become active and be able to bind the GHS-R1a (Kojima et al., 1999). This is a very well conserved modification, being found on vertebrates over millions of years (Kojima and Kangawa, 2005). Ghrelin is the only peptide currently known to suffer this modification. The responsible enzyme is the ghrelin O-acyltransferase (GOAT) (Yang et al., 2008a). The GOAT enzyme is located in the endoplasmic reticulum, where it octanoylates pro-ghrelin (Yang et al., 2008a).

Several ghrelin-like molecules have been isolated from the human stomach and plasma. These molecules differ on amino acid length and type of acylation; they include octanoyl ghrelin-(1-27), decanoyl (C10:0) ghrelin, decanoyl ghrelin-(1-27) and decenoyl (C10:1) ghrelin (Hosoda et al., 2003). Des-acyl ghrelin and des-acyl ghrelin-(1-27), without acylation, are present in human stomach and plasma. The des-acyl ghrelin plamatic concentration is in fact much higher than that of acylated ghrelin, with their ratio being respectively 1:10 in the case of rodents (Gauna et al., 2007) and 1:20 in humans (Marzullo et al., 2004); this suggests that after secretion, ghrelin is almost immediatly deacylated in the plasma (reviewed in (Satou et al., 2011)). Des-acyl ghrelin is an inactive form of ghrelin in that it does not bind the GHS-R1a nor stimulates GH release in vivo (Torsello et al., 2002). However, recent studies have started to unviel physiological and pathological functions of des-acyl ghrelin, arguing for a still undescribed role for this form of ghrelin on the ghrelin system (reviewed in (Delhanty et al., 2012)).

In terms of the regulation of ghrelin expression, it was observed that glucagon is capable of increasing stomach ghrelin mRNA levels on rats (Wei et al., 2005), suggesting that the increase in ghrelin plasma levels during food restriction may be due to the signaling by glucagon, which has its systemic levels increased during food restriction (Wei et al., 2005). This up-regulation of ghrelin may be related with an increased activity of the ghrelin promoter through the second messenger cAMP, which is in turn increased through activation of glucagon receptors present in endocrine cells of the gastric mucosa (Katayama et al., 2007; Wei et al., 2005). Leptin is also responsible for the regulation of ghrelin expression. Leptin inhibits ghrelin mRNA expression in stomach tissue culture prepared from rat (Zhao et al., 2008). Accordingly, administration of leptin directly into the rat stomach decreases ghrelin secretion (Kamegai et al., 2004). Curiously leptin increases ghrelin mRNA levels on mice stomach, if administered intraperitoneally for 5 consecutive days (Toshinai et al., 2001). In humans, leptin administration has no effect on ghrelin circulating levels (Chan et al., 2004). Other known important regulators of ghrelin secretion are glucose, insulin, somatostatin, growth hormone, melatonin, and the parasympathetic nervous system (Korbonits et al., 2004).

In terms of tissue distribution, ghrelin is primarily synthesized in the stomach from X/A-like cells, constituting ~20% of the endocrine cells in adult oxyntic glands (Date et al., 2000a). Gastrectomy in humans leads to an 80% drop on the plasma levels of ghrelin (Leonetti et al., 2003). The X/A-like cells can be divided in two types, the closed- and open-type, the latter being in contact with the gastric lumen (Sakata et al., 2002a). Luminal content is the primar regulator of the activity of the open-type cells, and

are also modulated mechanical distension, neural transmission and hormones (Solcia et al., 2000). A recent study, aimed at identifying the G protein-coupled receptor repertoire of gastric ghrelin cells, showed that these cells contain several Gαi/o and/or Gαq/11-coupled receptors inhibiting ghrelin secretion including the somatostatin receptors SSTR1, SSTR2 and SSTR3, the lactate receptor GPR81, the short-chained fatty acid receptor FFAR2, the long-chain fatty acid receptor FFAR4, and the calcium sensing receptor CasR; these cells also contain Gαs-coupled receptors capable of promoting ghrelin secretion, including the β1-adrenergic receptor, the gastric inhibitory polypeptide (GIP) and the secretin receptors, the composite receptor for the sensory neuropeptide CGRP (Ramp1 and Calcrl) and the melanocortin receptor MC4 (Engelstoft et al., 2013). Ghrelin secreting cells are also found in the duodenum, jejunum, ileum, and colon, with cells having decreasing capacity to produce ghrelin from the duodenum to the colon (Date et al., 2000a; Hosoda et al., 2000a; Sakata et al., 2002b). The pancreas also produces ghrelin during gestation, but after birth its ghrelin-producing capacity steeply decreases (Chanoine and Wong, 2004). During the prenatal period gastric levels of ghrelin are low, increasing after birth (Hayashida et al., 2002).

Ghrelin producing cells can also be found on the central nervous system. Ghrelin has been detected in the hypothalamic arcuate nucleus (Lu et al., 2002), a central hub for the regulation of food intake, and also in axons that innervate the lateral hypothalamus (LH), arcuate nucleus (ARC), and ventromedial (VM), dorsomedial (DM) and paraventricular nuclei (PVN) of the hypothalamus (Cowley et al., 2003). Ghrelin can also be found on pyramidal neurons of layer V in the somatosensory cortex and cingulate gyrus of rats. Ghrelin-containing neurons in the LH project to the dorsal vagal complex, as assessed by retrograde tracing (Hou et al., 2006). The capaticity of ghrelin to induce growth hormone release (Kojima et al., 1999) and the presence of ghrelin in the pituitary gland indicates that the regulation of growth hormone release by ghrelin may happen in an autocrine and paracrine manner (Korbonits et al., 2001). Ghrelin is also expressed in the placenta (Gualillo et al., 2001), testis (Tena-Sempere et al., 2002), lung (Volante et al., 2002), kidney (Mori et al., 2000), immune cells (Hattori et al., 2001a) and to have cyclic expression in the ovaries (Gaytan et al., 2003).

Human ghrelin is capable of being transported across the murine blood brain barrier (BBB) in both directions by a saturable system, in contrast mouse ghrelin is transported by saturable system only in the brain-to-blood direction (Banks et al., 2002). It was also observed that the transport of ghrelin across the BBB can be modulated by the metabolic status of the organism (Banks et al., 2008). Peripherally administered ghrelin activates hypothalamic neurons and increases food intake (Banks et al., 2002; Rüter et al., 2003). The recent work by Schaeffer and colleagues showed that plasmatic ghrelin binds neurons in the direct vicinity of fenestrated capillaries on the arcuate nucleus of the hypothalamus (Schaeffer et al., 2013). The vagal afferent pathway may also be responsible for the orexigenic effects of ghrelin (Gilg and Lutz, 2006) whereas the GH release effect may occur via the direct action of ghrelin on the hypothalamus (le Roux et al., 2005). Diano and co-workers have determined that human ghrelin is uptaken at different rates in different murine brain regions: the olfatory bulb and occipital cortex have the

highest uptake, followed by the hypothalamus, while the hippocampus and the rest of the brain had the lowest uptake (Diano et al., 2006). This study also demonstrated that the uptake of ghrelin into the hypothalamus and hippocampus can be saturated with unlabeled human ghrelin (Diano et al., 2006). Although ghrelin is capable of crossing the BBB, a recent work suggested that peripheral ghrelin may not mediate its effects through direct action on extra-hypothalamic regions, at least at a short term. Cabral and coworkers showed that peripherally administrated ghrelin mainly activates c-Fos in ARC neurons and that intra-cerebro-ventricullarly (ICV) administrated ghrelin activates c-Fos in a larger number of brain nuclei (Cabral et al., 2014). Moreover, ARC-ablated mice failed to increase food intake in response to peripheral ghrelin but fully responed to ICV administered ghrelin. This points towards a greater relevance of the central ghrelin secreting neurons, already described to be present in the hypothalamus (Hou et al., 2006).

Ghrelin secretion is mainly regulated by feeding: fasting causes the elevation and feeding causes decreases in the levels of ghrelin. Ghrelin plasma levels are also regulated by the acute and chronic nutritional status. Plasma ghrelin levels increase almost two-fold immediately before each meal, and fall to trough levels within 60-120 min after food intake (figure 2) (Cummings et al., 2001b). Levels of ghrelin are also found to be high during the night. The postprandial decrease of ghrelin levels is proportional to the ingested calorie load (Callahan et al., 2004). Evidence show that this regulation is exerted postgastrically (Williams et al., 2003). To date the mechanism of this postprandial fall in ghrelin levels is not known (Al Massadi et al., 2014). The orexigenic effect of ghrelin during fasting is the result of not only increase pulse frequency and amplitude of secretion but also of synchronized lower pulses of leptin secretion (Bagnasco et al., 2002). Fasting increases ghrelin expression only in the stomach, and not on the pituitary or hypothalamus (Torsello et al., 2003). It was observed that in humans, prolonged fasting for 3 days was incapable of changing the ghrelin levels in comparison to the baseline state suggesting that the meal-related changes are rather decreases after food intake than increases due to fasting (Chan et al., 2004). Nevertheless, it was also observed that caloric restriction in mice with a reduction of 60% of normal calories for 10 days resulted in a four-fold increase in circulating levels of ghrelin (Lutter et al., 2008). The preprandial increase in ghrelin levels correlates with hunger scores in humans initiating meals voluntarily, supporting a possible role of ghrelin in meal initiation (Cummings et al., 2004). Reviewed in (Al Massadi et al., 2014).

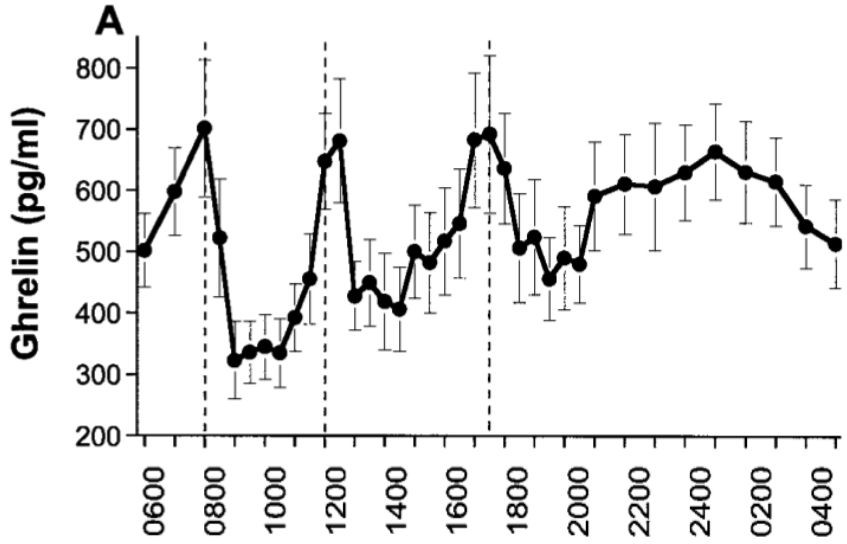


Figure 2 - Average plasma ghrelin concentration during a 24h period in 10 human subjects consuming breakfast (B), lunch (L), and dinner (D) at the times indicated (8.00h, 12.00h and 17.30h respectively). (adapted from (Cummings et al., 2001b))

Plasma ghrelin concentration is lower in obese people and higher in leaner people (Rosická et al., 2003). It is also higher in patients of anorexia nervosa, and this increase is reversed with the return to normal weight (Soriano-Guillen et al., 2004). There has been suggested a link between genomic variants of the ghrelin gene and obesity, namely Arg51Gln and Leu72Met. Obese patients with Met72 allele tend to become obese earlier in life. Arg51Gln mutation changes the C-terminal processing site of the ghrelin peptide within its percursor from Pro-Arg to Pro-Gln, resulting in a failure of the normal cleavage necessary to produce the 28 amino-acid ghrelin. A 94 amino-acid long pro-ghrelin peptide may still be produced, although its biological activity has not been assessed. Interestingly, it was reported in a Swedish study that the mutation Arg51Gln was identified specificaly in a group of obese patients (Kojima and Kangawa, 2005).

Gastrectomy leads to a reduction of circulating levels of ghrelin, and normal meal-related oscillations in ghrelin levels become absent (Leonetti et al., 2003). The absence of these oscillations is also seen in patients that undergone laparoscopic Roux-en-Y gastric bypass (LRYGBP) and laparoscopic adjustable silicone gastric banding (LASGB). Patients that undergone this two types of procedures had decreased levels of ghrelin relatively to controls, but yet increased levels in comparison with gastrectomized patients. A recent study by Grayson and co-workers reported that, in rats, Roux-en-Y gastric bypass (RYGBP) surgery leads to significantly increased levels of ghrelin in comparison with vertical sleeve gastrectomy, and these levels correlated with a greater improvement of cognitive function and hippocampal markers of inflammation after RYGBP on obese rats (Grayson et al., 2013). In patients with

short bowel syndrome, plasma ghrelin concentrations are also decreased probably due to loss of ghrelin-producing tissues (Krsek et al., 2003).

The phenotype of ghrelin knockout mouse shows normal size, growth rate, food intake, body composition, reproduction and gross behavior, without pathological changes. However, there is a trend towards lower body fat mass when the mice followed a high-fat diet (Kojima and Kangawa, 2005). The knockout of ghrelin also leads to a decrease in spine density on the CA1 region of the hippocampus and deficits on the novel-object recognition test (Diano et al., 2006), and to an increase in anxiety-like behavior response after restraint stress (Spencer et al., 2012). These results suggest that ghrelin is not a critical orexigenic factor but may function in nutrient sensing and switch of metabolic substrates. At the other end of the spectrum, a mouse model for ghrelinoma, a neuroendocrine tumor that releases great quantities of the ghrelin hormone, has been generated, using a ghrelin promoter SV40 T-antigen transgenic mouse (Iwakura et al., 2009). This mouse exhibits increased plasma ghrelin levels, activation of the GH-IGF-I axis and glucose intolerance (due to the suppression of insulin release). It would be interesting to further characterize this model in terms of behavior.

1.2.2 The Ghrelin receptor

The human ghrelin receptor, or growth hormone secretagogue receptor (GHS-R), is a prototypical seven transmembrane (TM) domain G protein-coupled receptor (GPCR) (reviewed in (Davenport et al., 2005)). It gene is located on chromosome 3 at 3q26.2, enconding the transcript 1a, a full-length receptor (GHS-R1a), and the transcript 1b, a shortened version (GHS-R1b) (McKee et al., 1997). The C-terminus of GHS-R1a is located in the intracellular region where it typically binds to G_q protein (Ferrini et al., 2009). Unlike GHS-R1a, the biological role of GHS-R1b is unknown and fails to bind growth homrone secretagogues (Howard et al., 1996). It has been suggested that this form of the receptor may modulate the function of the full-length receptor: both versions can coexist as heterodimers when expressed in HEK 293 cells, and GHS-R1b was shown to decrease the cell surface expression of GHS-R1a and inhibit its constitutive activity (Leung et al., 2007). This process occurs by the retention of GHS-R1a in the endoplasmic reticulum, by oligodimerization with GHS-R1b (Chow et al., 2012). GHS-R1b can form heterodimers with neurotensin receptor 1 (NTS1) and function as a receptor for neuromedin U, which can affect the growth of lung cancer cells (Takahashi et al., 2006).

GHS-R1a is a 366 amino acid polypeptide of ~41kDa (Camina, 2006), and is part of the family A of GPCRs. GHS-R1a is included in a family of receptors for small peptides comprising motilin (52% homology), neurotensin receptor-1 (NTS-R1) and NTS-R2 subtype (33-35% homology), neuromedin U receptor-1 (NMU-R1) and NMU-R2 subtype (~30% homology) and orphan receptor GPR39 (27-32% homology) (Tan et al., 1998). The ghrelin receptor is well conserved across vertebrates including chicken, pufferfish and a number of mammals (Palyha et al., 2000). This degree of conservation suggests that ghrelin and its receptor play important physiological roles.

Differences among the binding profiles of ghrelin, synthetic peptidyl and non-peptidyl growth hormone secretagogues (GHS), as are called molecules that bind GHS-Rs, is observed in tissues that either do not express GHS-R1a or express the receptor at low levels, suggesting the existence of an unidentified receptor (reviewed in (Muccioli et al., 2007)). Moreover, the existence of various GHS-R1a homologues, the existence of GHS-R1b, the absence of a clear phenotype for the GHS-R1a knockout, as well as the existence of vairous endogenous ghrelin-like ligands, points towards the existence of other ghrelin receptors (Muccioli et al., 2007).

The GHS-R1a can form heteromeric complexes with other GPCRs, namely dopamine receptor D1 and D2, melanocortin-3 receptor (MC3R) and serotonin receptor 2C (5-HT_{2C}) (Jiang et al., 2006; Kern et al., 2012; Rediger et al., 2012; Rediger et al., 2011; Schellekens et al., 2013), increasing the complexity of ghrelin signaling. Jiang and coworkers have shown that activation of GHS-R by ghrelin on neurons co-expressing dopamine receptor D1 (D1R) enhances dopamine/D1R signaling. This is achieved by switching G-protein coupled to the GHS-R from $G\alpha_{11/q}$ to $G\alpha_{i/o}$ by a mechanism consistent with agonist-dependent formation of GHS-R/D1R heterodimers (Jiang et al., 2006). GHS-R1a/dopamine receptor D2 (D2R) heterodimers were also found in hypothalamic neurons (Kern et al., 2012). The formation of these dimers modifies D2R canonical signaling resulting in $G\beta\gamma$ subunit-dependent mobilization of

intracellular calcium, independent of GHSR1a constitutive activity. The usage of a GHS-R1a antagonist was able to block the D2R agonist (cabergoline) induced anorexia. The ability of inhibiting D2R signaling with a GHSR1a antagonist is therapeutically relevant since neurons expressing D2R alone would not be affected (Kern et al., 2012). It was shown that GHS-R1a dimerization with unedited 5-HT_{2C}-INI receptor decreases GHS-R1a agonist-mediated calcium influx; this phenomenon is not with the partially edited 5-HT_{2C}-VSV isoform. The pharmacological blockade of the 5-HT_{2C} receptor restores this decrease(Schellekens et al., 2013). The 5-HT_{2C} receptor is crucial for reward-related behaviors (Alex and Pehek, 2007; Higgins and Fletcher, 2003), being functionally similar to the GHS-R1a in the homeostatic and hedonic regulation of food intake. Additionally, the neuronal 5-HT_{2C} receptor (Clemett et al., 2000; Leysen, 2004) is expressed on the same neuronal circuits expressing the GHS-R1a receptor (Cowley et al., 2003; Guan et al., 1997a; Zigman et al., 2006). 5-HT_{2C} receptor agonism and increases in brain serotonin levels was shown to inhibit the increase in plasmatic active ghrelin after overnight fasting in mice, supporting the existence of a negative feedback system (Nonogaki et al., 2006). The melanocortic receptor 3 (MC3R) induces increased food intake by influencing the hypothalamic control of body weight (Rediger et al., 2011). The melanocortin-induced intracellular cAMP accumulation was increased with coexpression of MC3R with GHS-R1a in comparison with the expression of MC3R alone. On the other hand, ghrelin-dependent and constitutive activity of GHS-R1a are disrupted by this interaction. It was also shown that MC3R signaling depends directly on the activity of GHS-R1a (Rediger et al., 2011).These findings suggest that GHS-R1a activity may play a role on the fine-tuning of neurotransmitter systems, already known to regulate mood and cognition, and neuropeptide systems.

In terms of distribution, the ghrelin receptor expression is widespread throughout the brain. GHS-R1a mRNA is detectable in various hypothalamic nuclei and the pituitary gland (Zigman et al., 2006), as expected for ghrelin to be able to modulate energy metabolism and GH release. GHS-R1a can also be found on cerebral cortex and regions of the midbrain, including ventral tegmental area and substantia nigra, and also on the pons, medulla oblongata and hippocampus (Guan et al., 1997a; Howard et al., 1996; Zigman et al., 2006). The GHS-R1a is found on presynaptic boutons in the arcuate nucleus, lateral hypothalamus, paraventricular nucleus of the hypothalamus and cortex (Cowley et al., 2003). The recent work by Mani and colleagues aimed at characterizing the brain expression of GHS-R1a, through the usage of a transgenic mice with an eGFP transgene regulated by the ghsr promoter. eGFP-immunoreactivity was present on the prefrontal cortex, insular cortex, olfatory bulb, amygdala and hippocampus. However, the eGFP levels were unexpectably low in midbrain regions and hypothalamic nuclei where GHSR expression is already well characterized, putting into question the validity of this model (Mani et al., 2014).

In the hippocampus GHS-R1a mRNA is detectable in CA1, CA2 and CA3 subregions and dentate gyrus (Zigman et al., 2006). GHS-R1a is present both in the cell soma and dendrites in the CA1, and shows a punctate pattern of labeling along dendritic shafts (Berrout and Isokawa, 2012). A large fraction of GHS-

R1a localizes to excitatory synapses in rat hippocampal neurons (Ribeiro et al., 2014). The mRNA expression is undetectable in the fetal rat hippocampus, and only becomes detectable after postnatal day 7 (P7), (Ribeiro et al., 2014). On the rest of the body, the ghrelin receptor is widely distributed, being found in adipose tissue, myocardium, gonads, lungs, liver, arteries, stomach, pancreas, and thyroid, among other sites (Ferrini et al., 2009).

Upon ghrelin binding to GHS-R1a the intracellular loops of the receptor change conformation and expose G protein binding site. The coupling of G proteins to GHS-R1a activates G protein subunits and induces the transduction of the receptor signaling to intracellular effectors (reviewed in (Gao and Horvath, 2007; Muccioli et al., 2007)). Activated GHS-R1a couples primarily, but not only, to Gαq/11, leading to the activation of phosphatidylinositol-specific phospholipase C (PI-PLC), that generates inositol 1,4,5-triphosphate (IP_3) and diacylglicerol (DAG) from phosphatidylinositol 4,5-diphosphate (PIP_2) (Howard et al., 1996; Smith et al., 1997), represented on figure 3. IP_3 mobilizes cytoplasmic storage pools of calcium (Ca^{2+}), increasing the free cytoplasmic Ca^{2+} concentration in a rapid manner. Protein kinase C (PKC) is activated by calcium and DAG. PKC inhibits plasma membrane potassium channels causing membrane depolarization (Camina, 2006). A more sustained accumulation of intracellular Ca^{2+} is achieved after the opening of voltage-dependent L-type Ca^{2+} channels (Korbonits et al., 2004), which by itself also contributes for the membrane depolarization (Figure 3). This signaling is observed, for instance, on the ability of GHS-R1a to enhance firing of nigral dopaminergic neurons through the inhibition of voltage-gated potassium Kv7/KCNQ/M-channels, which is signaled by the activation of the PLC-PKC pathway (Shi et al., 2013). GHS-R1a also couples to $G_{i/o}$ which, in addition to its canonical coupling to G_q, is crucial to signal downstream effectors like MAP kinase (MAPK) and PI3 kinase (PI3K), that where already shown to mediate cellular proliferation in several cell populations (Camina, 2006; Kim et al., 2004; Mazzocchi et al., 2004). GHS-R1a is also capable of coupling to G_s in NPY-producing neurons in the hypothalamus, enabling the increase in intracellular Ca^{2+} concentration through N-type Ca^{2+} channels in these neurons (Kohno et al., 2003). Additionally, GHS-R1a downstream effectors include the nitric oxide/cyclic guanosine monophosphate (cGMP) pathway, which is crucial for inducing GH release on porcine somatotropes (Rodriguez-Pacheco et al., 2006), the 5'-AMP-activated protein kinase (AMPK) in the hypothalamus (Andersson et al., 2004) and the G_q-Calmodulin-dependent protein kinase IV (CaMKIV)-cAMP responsive element binding protein (CREB) pathway (Holst et al., 2003). Ghrelin is therefore capable of coupling several G-protein subunits and activating diverse signaling effectors; this provides an immense diversity of signaling possibilities, determined by the cell population's molecular characteristics.

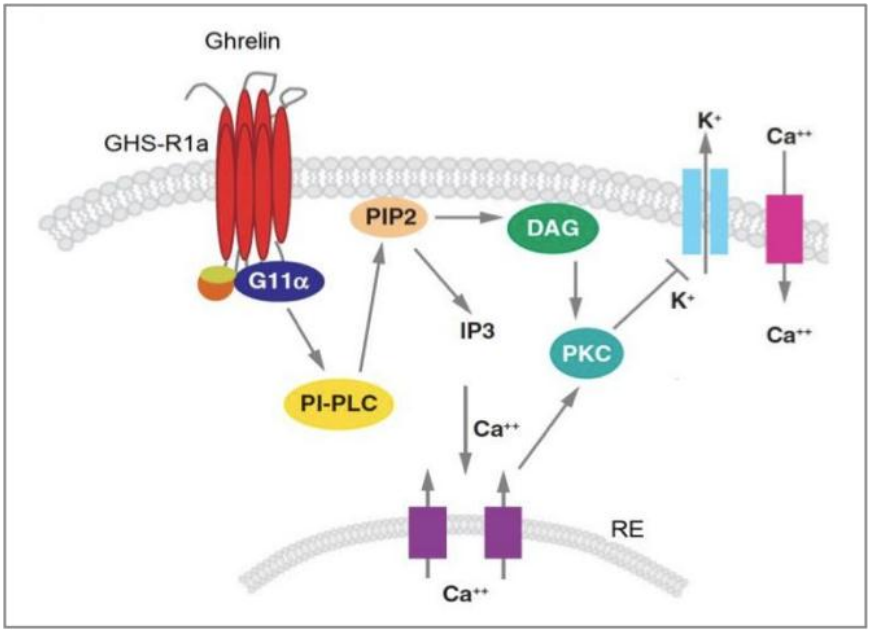


Figure 3 - Prototypical signaling of GHS-R1a. Ligand binding activates $G_{aq/11}$ protein that signals phosphatidylinositol-specific phospholipase C (PI-PLC), which hydrolyzes phosphatidylinositol 4,5-diphosphate (PIP_2), stored in the plasma membrane, generating both diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 will induce the rapid release of Ca^{2+} stored in the endoplasmic reticulum, and the free Ca^{2+} together with DAG will activate PKC. PKC will inhibit K^+ channels, leading to a membrane depolarization and causing the opening of voltage-dependent L-type Ca^{2+} channels (Adapted from (Gao and Horvath, 2007))

GHS-R1a has also the remarkable property of displaying significant constitutive activity (Holst et al., 2003), which can be demonstrated using an identified inverse agonist for the receptor, (D-Arg1,D-Phe5,D-Trp7,9,Leu11)-substance P (Holst et al., 2003; Holst et al., 2006; Petersen et al., 2009). This constitutive activity signals similar pathways to the ligand-dependent activity: PLC pathway, CREB pathway and serum responsive element (SRE) pathway (Holst et al., 2004). This constitutive activity achieved by the presence of three aromatic residues: PheVI:16 (phenylalanine at position 16 in transmembrane domain 6), PheVII:6 and PheVII:9. The extracellular end of TM7 (transmembrane domain 7) is docked into TM6 through the presence of these hydrophobic residues, and so enabling the receptor to maintain an active conformation (Holst et al., 2004). The constitutive activity is relevant for the expression of NPY and food intake, as shown by the observation that intracerebroventricular injection in rats of the inverse agonist decreases feeding and body weight (Petersen et al., 2009). The levels of phosphorylated CREB in a hypothalamic cell line were reduced after administration of the inverse agonist and higher with administration of ghrelin. It was remarkable that the inverse agonist, which is unable of reduce food intake increased by ghrelin, was capable of reducing feeding and preventing body weight gain observed in the control group (Petersen et al., 2009) (reviewed in (Mear et al., 2013)). The Ala204Glu and Phe279Leu GHS-R1a polymorphisms have been linked to obesity and

short stature, respectively (Wang et al., 2004). Phe279 is essential for the receptor to maintain its active conformation (Holst et al., 2004) and Ala-204 is crucial for its constitutive activity, for when GHS-R1a carrying the mutation Ala204Glu is expressed in HEK-293 cells, both the consitutive activity and surface expression of the receptor is reduced (Pantel et al., 2006). Loss of constitutive activity can thus be important on pathologies linked to short stature and obesity (Holst and Schwartz, 2006).

The GHS-R knockout mice show an absence of typical increases in GH release and food intake upon ghrelin administration. Growth, development, appetite and body composition are normal suggesting that ghrelin and its receptor are not critical for growth and appetite regulation or alternatively that if not present during development, the lack of GHS-R may be compensated by other signaling mechanism. However, plasmatic insulin-like growth factor I (IGF-I) levels and body weight were shown to be reduced, which also suggests that ghrelin may regulate the levels of IGF-I for the maintenance of an anabolic state (Kojima and Kangawa, 2005).

1.3 GHRELIN SYSTEM PHYSIOLOGY

1.3.1 Regulation of food intake

Feeding is essential for an individual's survival. Ghrelin and its receptor, GHS-R1a, play a central role in the regulation of food intake. Ghrelin is capable of stimulating appetite by increasing not only food intake itself but also the rewarding aspects of food intake. Ghrelin regulates homeostatic and hedonic aspects feeding: indeed, abnormalities of the ghrelin system are observed in several eating disorders, such as, obesity, anorexia nervosa, cachexia and Prader-Willi syndrome (Portelli and Smolders, 2014). Homeostatic feeding takes place with there is a shortage of nutrients, the fuel stores become depleted and the levels of ghrelin are elevated. Hedonic feeding is motivated by cognitive, reward and emotional factors that contribute to the consumption of pleasurable foods, even when this caloric intake is unnecessary from a homeostatic point of view. The neural circuits controlling homeostatic feeding are located in the brainstem and hypothalamus, while the circuits controlling hedonic feeding are mainly related with cortico-limbic structures (hippocampus, amygdala, cortex and the mesocorticolimbic dopamine circuit) (reviewed in (Williams and Elmquist, 2012)).

In the medial basal region of the hypothalamus, melanocortin plays a central role on the neuronal control of homeostatic feeding. This circuit consists of two subsets of neurons located in the arcuate nucleus of the hypothalamus (ARC), represented on figure 4. These neurons have access to various humoral signals that can access the hypothalamus through fenestrated capillaries at its base, express receptors for a great variety of signaling molecules and respond rapidly to energy balance changes. The two subsets of neurons found on ARC have opposite effects on feeding, but are both GABAergic. One set expresses pro-opiomelanocortin (POMC) and cocaine-amphetamine regulated transcript (CART) and when stimulated produce anorectic effects. POMC precursor is cleaved to give rise to melanocyte-stimulating hormones (α-, β- and γ-MSH), β-endorphin and adrenocorticotrophic hormone (ACTH). Of these, α- and β-MSH reduce food intake and body weight and increase energy

expenditure in animals and humans. Both these hormones act on melanocortic receptor subtypes 3 and 4 (MC3/4R), which are abundant in the ARC, PVN, LH and DM. The second set of neurons lead to orexigenic responses and contain neuropeptide Y (NPY) and agouti gene-related transcript (AgRP). NPY stimulates feeding and reduces energy expenditure. AgRP is also orexigenic, acting as an antagonist of MC3/4R, reducing the anorectic effects of α-MSH. The POMC and NPY neurons in the ARC have projections to various brain nuclei, including the PVN, LH and perifornical hypothalamic region, all of which have substantial numbers of MC3/4R. These second-order neurons project then to other areas involved in the regulation of food intake, including the dorsal vagal complex in the brainstem, comprised of the nucleus tractus solitarius (NTS), the area postrema (AP), and the dorsomotor nucleus of the vagus (DMV). The dorsal vagal complex also senses peripheral signals and integrates neural inputs from vagal afferent fibers that transmit visceral information including gastric distension and gut factors (Williams and Elmquist, 2012). Circulating ghrelin increases the rate of firing of NPY neurons and diminishes the frequency of action potentials of POMC cells, by acting on presynaptic GHS-R1a receptors. In contrast with ghrelin, plasma leptin increases the firing of POMC cells via pre- and postsynaptic modes of action and inhibits the firing of NPY cells (reviewed in(Gao and Horvath, 2007)). Thus ghrelin and leptin have opposite roles in feeding regulation. Remarkably, ghrelin is the only known orexigenic hormone from the periphery to stimulate food intake.

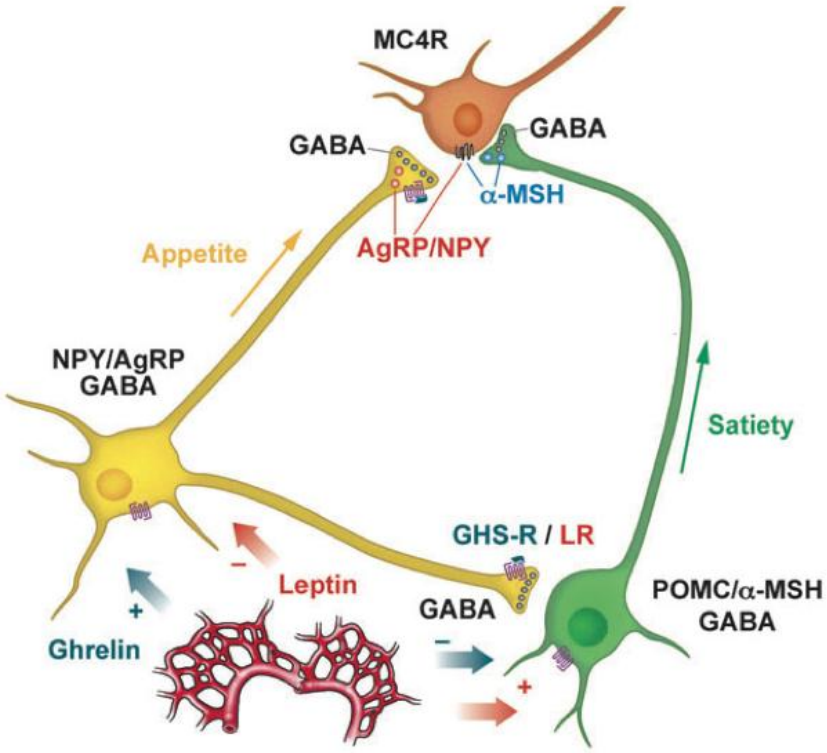


Figure 4 - Schematic illustration of the melanocortin system. It consists of two subsets of ARC neurons: the POMC and NPY neurons. The release of POMC-driven α-MSH at target sites, which binds melanocortin 4 receptor (MC4R), promotes satiety and is the result of signaling of POMC cells by leptin. Agouti-related peptide (AgRP) is also expressed by ARC NPY neurons, which are inhibited by leptin and stimulated by ghrelin. Activation of these neurons results in an increased food intake, decrease in energy expenditure and the silencing of satiety-promoting POMC neurons. The NPY cells activation results on the release of GABA, NPY and AgRP, the latter being an inverse agonist of MC4R, thus antagonizig the anorexigenc effects of α-MSH. (adapted from (Gao and Horvath, 2007))

Apart from circulating ghrelin acting on hypothalamic neurons, ghrelin-expressing neurons in the hypothalamus have also an important role on the regulation of appetite (Cowley et al., 2003). In the ARC, these neurons synapse NPY- and AgRP-expressing neurons and stimulate the release of these orexigenic peptides and the GABA-mediated inhibition of POMC-containing neurons. The PVH NPY neurons also recieve fibers from hypothalamic ghrelin expressing neurons (Cowley et al., 2003).

Ghrelin also regulates food intake by directly signaling brainstem nuclei. In the brain stem, the area postrema and adjacent nucleus of the solitary tract have receptors for various metabolic hormones including cholecystokinin (CKK), estrogen, leptin and ghrelin. ICV ghrelin infusions increase the expression of Fos on these regions (Lawrence et al., 2002). These studies also showed an increase in food intake after infusion of ghrelin into the fourth ventricle or the dorsal vagal complex increases food intake (reviewed in (Abizaid and Horvath, 2012)).

The effect of ghrelin on hedonic feeding will be reviewed on the food reward section.

1.3.2 Adiposity

Plasma ghrelin concentration is higher before food ingestion and during caloric restriction, and decreases postprandially. Considerable evidence points towards the role of ghrelin in the sensation of hunger and initiation of meals (Lutter et al., 2008; Tschop et al., 2001). In rodents, ghrelin increases weight by increasing fat mass gain and reducing fat utilization (Tschop et al., 2000).

Ghrelin is involved in certain types of human obesity (reviewed in (Hillman et al., 2011)). Gastric bypass surgery is known to decrease ghrelin plasmatic levels. Several studies have also identified a decrease in ghrelin levels after weight loss aided by gastric bypass in comparison to baseline levels. Remarkably, diet induced weight loss does not result in a decrease in the levels of ghrelin, and may even increase ghrelin levels, as occurs in other situations of energy insufficiency (reviewed in (Cummings et al., 2005)). This decrease of ghrelin levels may favor gastric bypass as an effective media to achieve long-term weight loss. While in obese patients the baseline plasmatic concentration ghrelin may be decreased

relatively to healthy controls, in Prader-Willi syndrome ghrelin levels are significantly elevated. It has been suggested that these elevated ghrelin levels may be responsible for the persistent hyperphagia observed on these patients (Hillman et al., 2011). Additionally, a study by Gjesing and colleague's found a rare promoter variant that partially co-segregates with obesity and overweight in two different Danish pedigrees (Gjesing et al. 2010).

Pharmacologically modulating ghrelin signaling may reduce food intake and contribute to weight loss (Hillman et al., 2011). Reduction of bioavailable ghrelin or daily administration of a GHS-R1a antagonists was already shown to reduce food intake in diet-induced obese mice and promotes a decrease in body weight (Esler et al., 2007; Rudolph et al., 2007). In line with this, an anti-obesity vaccine has been developed to prevent ghrelin from accessing the central nervous system (Schellekens et al., 2010). Other pharmacologic approaches also include ghrelin enantiomers that can neutralize ghrelin, and decreasing acyl ghrelin levels through inhibition of GOAT. Nevertheless, presently there is no anti-ghrelin drug on the market mainly because of their reduced efficacy, insufficient selectivity, and poor bioavailability (reviewed in (Hillman et al., 2011)).

1.3.3 Anxiety and Depression

Various neuropeptides implicated on homeostatic energy balance are also involved in anxiety-like behavior. The link between food intake and anxiety regulation may lie on neuropeptide-regulated defensive survival mechanism being an evolutionary adaptation for the regulation of responses to threats in the environment (Bowers, 2012). For example, NPY has both anxyolitic and orexigenic effects (Heilig et al., 1989), cholecystokinin, apart from being involved on nutrient metabolism, induces panic-like effects (Strohle et al., 2000) affects general anxiety, stress-related behavior and enhances attention and memory (Dauge and Lena, 1998), and neuropeptide S has both anorexigenic (Smith et al., 2006) and anxiolytic effects (Xu et al., 2004).

Ghrelin administration, via intracerebroventricular (ICV) or intraperitoneal (IP) injections, has been reported to induce anxiety-like behavior in mice and rats, as determined by the elevated plus maze (Asakawa et al., 2001; Carlini et al., 2002a; Kajbaf et al., 2012). Furthermore, knockdown of ghrelin through the administration of an anti-sense oligonucleotide produced anxiolytic and antidepressant effects in rats (Kanehisa et al., 2006). ICV administration of ghrelin to chicks was also shown to induce anxiogenesis (Carvajal et al., 2009). These findings support the notion that ghrelin has anxiogenic effects by activating the hypothalamic-pituitary-adrenaline (HPA) axis and inducing the release of corticotrophin-releasing hormone (CRH) in the PVN. GHS-R1a is expressed at high levels in the PVN, which supports a role for GHS-R1a on the secretion of CRH. Indeed, IP injections of ghrelin in mice increase hypothalamic CRH mRNA in a dose-dependent manner (Asakawa et al., 2001). Microinjections of ghrelin into the ARC, PVN, VMN and perifornical hypothalamus (PFH), in rats, also increases anxiety-like behavior as assessed by elevated-plus maze. Additionally, the ARC and PVN were sensitive

to lower amounts of ghrelin when comparing to the other brain regions, indicating an increased sensitivity of these hypothalamic regions to ghrelin (Currie et al., 2012). Apart from the hypothalamus, ghrelin most potently increases anxiety-like behavior in the dorsal raphe nucleus (DRN), which is the major site for serotonergic neurons in the brain (Carlini et al., 2004b). This fact suggests that the anxiogenic effects are also the result of modulation of the serotonergic system. Furthermore, injections of ghrelin into the basolateral amygdala of rats induced increase in anxiety, as assessed by elevated-plus maze (Currie et al., 2014). Finally, in rats ICV administered with ghrelin chronically (> 2weeks), there was an increase in anxiety- and depression-like behavior (Hansson et al., 2011), which was accompanied by an increased amygdalar expression of corticotrophin releasing hormone receptor 1 (Crhr1) mRNA. Crhr1 is associated with increased anxiety-like behavior in stressful contexts, such as social defeat (Liebsch et al., 1995). In their experiments, Hansson and colleagues also reported that 5-HT_{1A}-receptor mRNA expression was increased in the amygdala (Hansson et al., 2011). This is in concordance with previous observation on the involvement of the serotonin system in ghrelin's effects (Carlini et al., 2007; Carlini et al., 2002a; Carlini et al., 2004b).

Disputing the aforementioned conclusions, ghrelin was also shown to have anxiolytic (Alvarez-Crespo et al., 2012; Lutter et al., 2008; Spencer et al., 2012) and anti-depressive effects (Lutter et al., 2008). The work by Lutter and colleagues showed that increasing ghrelin through caloric restriction or subcutaneous injection decreased anxiety- and depressive-like behavior, as evaluated by elevated plus maze and forced swim tests, respectively. These effects were specific for GHS-R1a signaling, as they were absent in the GHS-R1a knockout. In the same study it was also suggested that the antidepressant effects of ghrelin include the stimulation of orexin neurons in the LH, as mice without these oxerin neurons did not show the antidepressive-like effects of ghrelin in the forced swim test (Lutter et al., 2008). Acylated ghrelin levels were enhanced following chronic social defeat stress (CSDS) in mice, a model of prolonged psychosocial stress, and social avoidance was increased in GHS-R1a null mice, suggesting that ghrelin regulates social isolation in response to CSDS (Lutter et al., 2008). In another study carried out by Spencer and co-workers, supporting this opposing hypothesis, it was shown that ghrelin knockout mice were more anxious after acute restraint stress. Additionally, the authors found evidences that ghrelin reduces anxiety after acute stress by stimulating HPA axis at the level of the anterior pituitary (Spencer et al., 2012). Alvarez-Crespo and coworkers showed that by directly injecting ghrelin into the amygdala of rats, ghrelin produced anxyolitic-like effects only if the animal were food-restricted for 1 hour between ghrelin injection and behavioral tests (open-field and elevated-plus maze) (Alvarez-Crespo et al., 2012). Wistar Kyoto rats (which are more anxious than other rat strains) have lower plasma levels of ghrelin than Sprague Dawley rats (Kristensson et al., 2007), arguing in favor on an axyolitic effect of ghrelin. Stress leads to the elevation of ghrelin plasmatic levels on both Wistar Kyoto and Sprague-Dawley rat strains, but these elevations are significantly lower in the anxiety prone Wistar Kyoto animals than in the Sprague-Dawley animals (Kristensson et al., 2007).

Table 1 shows a summary of the characteristics of all the major published works analyzing the effects of ghrelin on anxiety.

Table 1 – Main evidence for ghrelin effects on anxiety and the characteristics of the experiments. ICV, intracerebroventricular. IP, intraperitoneal. SC, subcutaneous; OF, open-field. EPM, elevated-plus maze, DLB, dark-light box;

Reference	Ghrelin effect on anxiety	Effective drug dose and administration route	Species and Strain of animals used	Age of the animals and weight	Number of animals per condition	Behavioral test	Time between administration and test	Special Remarks
Asakawa et al., 2001	Anxiogenesis	0.3 and 1 nmol of ghrelin ICV and 0.3-3 nmol IP, Ghrelin	ddy mice	7 weeks old	From n=10 to n=13	EPM	10 minutes	
Carlini et al., 2002	Anxiogenesis	1.5 and 3 nmol ICV, Ghrelin	Male Wistar rats	not mentioned (240-260g)	From n=6 to n=10	OF and EPM	10 minutes	
Carlini et al., 2004	Anxiogenesis	0.3, 1.5 and 3 nmol injected into various brain regions, Ghrelin	Male Wistar rats	Not mentioned (240-260g)	From n=5 to n=12	EPM	Not mentioned	
Kanehisa et al., 2006	Anxiogenesis*	0.5 µg of antisense DNA for GHS-R ICV, constantly for 3 days	Male Wistar rats	7-9 weeks old (307-329g)	Form n=9 to n=11	EPM and Adapted DLB	Anxiety tests performed on the first day after surgical DNA infusion	Antisense DNA for GHS-R induce decreases in anxiety-like behavior
Carvajal et al., 2009	Anxiogenesis	0.08 and 0.3 nmol ICV, Ghrelin	Chicks (young *Gallus gallus domesticus*)	Few hours old	From n=9 to n=10	OF	Immediately (?)	
Hansson et al., 2011	Anxiogenesis	3 pmol per day injected ICV with osmotic mini pump for 29 days, Ghrelin	Male Sprague-Dawley rats	Not mentioned (270-300g)	n=11	OF, EPM, DLB and social interaction test	Not mentioned	
Kajbaj et al., 2012	Anxiogenesis	3 nmol ICV, GHPR-2 ghrelin receptor agonist	Female Wistar rats, sham and ovarectomized	not mentioned (180-240g)	n=10	EPM	10 minutes	
Currie et al., 2012	Anxiogenesis	0.2, 0.4 and 0.8 nmol injected into various hypothalamic nuclei, Ghrelin	Male Sprague-Dawley	Adult (275-315g)	From n=12 to n=14	EPM	10 minutes	
Currie et al., 2014	Anxiogenesis	0.3 nmol injected into the basolateral amygdala, Ghrelin	Male Sprague-Dawley rats	Adult (275-300g)	From n=10 to n=12	EPM	5 minutes	
Lutter et al., 2008	Anxyolitic	0.6 pmol per g of animal weight injected SC, Ghrelin	C57BL6/J mice	8-10 weeks old (weight not mentioned)	n=8	EPM	45 minutes	
Alvarez-Crespo et al., 2012	Anxyolitic*	0.3 pmol per g of animal weight injected directly into amygdala, Ghrelin	Male Sprague-Dawley rats	Adult (200-250g)		OF and EPM	1 hour, without access to food	*No effects on anxiety were observed if animals had access to food during the waiting hour
Spencer et al., 2012	Anxyolitic	*0.3 pmol per g of animal weight SC, Ghrelin	C57BL6 background mice with -/- GHS-R	8-10 weeks old (weight not mentioned)	From n=3 to n=10	OF, EPM before and after restraint stress	*30 minutes, plus 15 minutes of restraint stress, plus 120 before perfusion	*Knockout mice showed decreased anxiety after restraint stress and ghrelin recovered hypothalamic c-fos expression on the knockouts

To reconcile these observations, it was suggested that the disparity of results could be related to the timing of the behavioral tests after ghrelin administration (Andrews, 2011). The majority of the studies that demonstrated anxiogenic effects of ghrelin conducted behavioral tests within 10 min of administration (Asakawa et al., 2001; Carlini et al., 2002a; Carlini et al., 2004b; Currie et al., 2012; Kajbaf et al., 2012; Kanehisa et al., 2006)(or within 5 minutes (Currie et al., 2014)), whereas the study showing anxyolitic-like effects conducted behavioral tests 45 min or more after administration (Alvarez-Crespo et al., 2012; Lutter et al., 2008). Since ghrelin has a plasmatic half-life of ~30 min, it is arguable that the bolus dose, which potently induces food intake in several studies (Asakawa et al., 2001; Carlini et al., 2002a; Carlini et al., 2004b; Kanehisa et al., 2006), also induces the anxiogenic responses. Other factors influencing these results may be: different types and durations of stressors (Schellekens et al., 2012), route of administration of ghrelin, timing of administration, strain or species used, nutritional state of the animals (Alvarez-Crespo et al., 2012; Lutter et al., 2008) or other experimental details such as handling of the animals (Schellekens et al., 2012). The clarification of this issue is an objective of this thesis.

Circulating levels of ghrelin have been shown to increase in conditions of stress in both rodents and humans (Schellekens et al., 2012). It is however unclear if ghrelin may be functioning in reaction to stress or as a component of the stress response. Cold exposure, acute fasting, caloric restriction, as well as psychological stress, are capable of increasing ghrelin levels. In rodents tail pinch and water avoidance stress protocols have also been shown to increase gastric ghrelin mRNA levels and total plasma ghrelin (Asakawa et al., 2001; Kristenssson et al., 2006). Interestingly, reduced plasma ghrelin levels were observed following physical stressors, such as endotoxin injection, abdominal surgery and exercise (Stengel et al., 2011). Although the pathways underlying the changes in ghrelin plasma concentration following different stressing agents are still largely unknown, it has been suggested that they are mediated via a sympathoadrenal response subsequent to the activation of the sympathetic nervous system and catecholamine release (Mundinger et al., 2006; Zhao et al., 2010). In GHS-R1a null mice stress-induced metabolic changes, such as decreased caloric intake and decreased body weight, were absent (Patterson et al., 2010). The increase in ghrelin may potentially shed light on the phenomenon of "comfort eating" in conditions of stress. In humans, circulating levels of plasma ghrelin correlate with cortisol levels following the standardized Trier-Social-Stress test (Rouach et al., 2007). In humans and in rats it was also observed that ghrelin administration increases cortisol and adrenocorticotropic hormone secretion (Arvat et al., 2001; Ochi et al., 2008; Takaya et al., 2000).

Regarding the effects of ghrelin on mood, the mechanism behind these effects may be related with the aforementioned effects of ghrelin on orexin neurons as well as the effect of ghrelin on

the VTA (further explored on the "Food Reward" section), its effects on the hippocampus (further explored on the "Cognitive effects of ghrelin" section) and the anti-inflammatory properties of ghrelin. It has been recognized that hippocampal circuitry together with the neocortex mediates the several of the cognitive aspects involved in depression, such as memory impairment and feelings of worthlessness, hopelessness, guilt and suicidal tendencies (Nestler et al., 2002). Ghrelin has also been shown to stimulate cellular proliferation and differentiation of adult rat hippocampal progenitor cells (Li et al., 2013a; Li et al., 2013b; Moon et al., 2009), suggesting that ghrelin might also be involved in hippocampal neurogenesis. Neurogenesis itself is thought to underlie the therapeutic action of several antidepressants (Sahay and Hen, 2007). This property of ghrelin may also be important for its cognitive effects (see section "Cognitive Effects"). Brain inflammation is currently being explored in connection with psychiatric disorders (reviewed in (Dantzer et al., 2008; Raison et al., 2006)). Several studies have shown that administration of cytokine or cytokine inducers such as LPS may lead to the development of depression, while anti-inflammatory treatments generate antidepressant effects (Tyring et al., 2006). GHS-Rs have been found to be expressed in immunocytes (Hattori et al., 2001a), as previously mentioned, and ghrelin and ghrelin mimetics also have been shown to display immunosuppressive actions via the inhibition of proinflammatory cytokines (Dixit et al., 2004; Granado et al., 2005; Himmerich and Sheldrick, 2010; Li et al., 2004). Therefore it is hypothesized that stress-induced elevations in ghrelin may help alleviate damage caused by inflammation within the brain (Chuang and Zigman, 2010).

Polymorphisms in the ghrelin gene have also been associated with depression in humans (Nakashima et al., 2008) and ghrelin levels were found to be decreased in patients suffering from depression (Barim et al., 2009). However, these changes in ghrelin levels are not consistantly observed (Kluge et al., 2009; Schanze et al., 2008). Interestingly, ghrelin administration may display some antidepressant effects when given to patients with major depression (Kluge et al., 2011). Nonetheless, the relationship between depression and ghrelin is a subject requiring further exploration.

1.3.4 Food reward

Food palatability and caloric content increase the non-homeostatic motivation to obtain food and the resulting over-consumption is central to the pathology of obesity (Schellekens et al., 2012). Recent evidences suggest that ghrelin is an important signaling hormone not only on homeostatic but also on hedonic food intake. GHS-R1a is highly expressed at the VTA, supporting a role for ghrelin in VTA-mediated reward signaling (Schellekens et al., 2012). Projections from VTA that terminate in the nucleus accumbens (NAcc) and the prefrontal cortex

play a significant role in anticipatory food reward and food-seeking behavior (Schellekens et al., 2012). Indeed ghrelin is a key mediator in hedonic feeding: it has been shown that ghrelin infused into the VTA produces an overflow of dopamine within the NAcc (Jerlhag et al., 2007); direct microinjection of ghrelin both in VTA or in the NAcc strongly enhances feeding behavior (Abizaid et al., 2006); icv administered ghrelin enhances preference for rewarding foods, increases fat ingestion (Shimbara et al., 2004), and stimulates increased consumption of saccharin solutions in wildtype mice, but not in GHS-R1a knockout mice (Disse et al., 2010). Following central ghrelin administration in GHS-R1a knockouts or upon treatment of rats with GHS-R1a antagonist, the intake of palatable food is decreased in a free choice paradigm (normal chow versus appetitive food) (Egecioglu et al., 2010). Moreover, it has already been observed a ghrelin-dependent food conditioned place preference (Disse et al., 2011; Egecioglu et al., 2010). More recently, intra-VTA ghrelin injections not only increased free feeding of chow but also enhanced behavior leading to a sucrose reward (Skibicka et al., 2012). In contrast, while regular chow intake was intact, stimulation or blockade of the GHS-R1a receptor did not affect NAcc mediated eating behavior (Skibicka et al., 2012). Recently, Cone and colleagues showed that lateral ventriculum infusion of ghrelin increases dopamine spikes in the NAcc after food intake (Cone et al., 2014). This effect was also observed when ghrelin was injected to the lateral hypothalamus (LH) but surprisingly, not when ghrelin is injected directly into the VTA. The authors proposed that VTA-projecting LH orexin neurons could be activated by ghrelin and responsible for the observed effect (Cone et al., 2014). In sum, these results point to the VTA, but not the Nacc, as a direct target site for ghrelin's action on food-motivated behavior. Whereas ghrelin may increase appetite via the NAcc, in the VTA it affects the reward and motivational aspects of food intake (Dickson et al., 2011; Skibicka and Dickson, 2011). In humans, ghrelin enhances the activity in the brain reward centers induced by appealing food pictures (Malik et al., 2008). Ghrelin is also lowers threshold dose of cocaine necessary to induce conditioned place preference and mediates alcohol reward (Davis et al., 2007; Jerlhag et al., 2009). It is now clear that ghrelin is an important part of the physiology and pathology of the brain reward centers. Its action on these circuits is also a key to understanding its effects on cognition.

1.3.5 Cognitive effects

The ability to locate food sources, remembering those locals and recalling whether or not all the available food was consumed are evolutionarily important survival skills (Moran and Gao, 2006). This raises the possibility that these behaviors may be regulated by signaling molecules, such as ghrelin, involved in energy balance. Indeed ghrelin-mediated improvements in memory performance have recently gained attention (Diano et al., 2006).

The work by Carlini and co-workers in 2002 was the first to show that ghrelin enhances memory retention in a dose-dependent manner when given immediately after training, suggesting that it promotes improvements in memory retention (Carlini et al., 2002a). The researchers subsequently applied intraparenchymal injections of various amounts of ghrelin in the hippocampus, dorsal raphe nucleus and amygdala finding that the dose-dependent maximal improvement of retention was achieved by adiministration of ghrelin into the hippocampus (Carlini et al., 2004b). More specifically, dorsal hippocampus infusion of ghrelin in rats led to a spatial memory enhancement in the water maze test (Chen et al., 2011). It was also detected that intra-hippocampal ghrelin administration before the training sessions of step-down avoidance test improved long-term memory of the task, without affecting short-term memory. No performance changes were observed with ghrelin administration prior to the test session. These observations argue in favor of ghrelin affecting specifically memory retention mechanisms (Carlini et al., 2010). Spontaneous alternation in plus-maze task was also improved when ghrelin was administered before the test (Diano et al., 2006). In the same study, by Diano et al., an increased retention was observed if T-maze foot-shock and step-down passive avoidance tests were conducted after ghrelin administration, supporting the aforementioned observations by Carlini and co-workers. In 12- and 14-month old SAMP8 mice, which mimic some of the Alzheimer disease cognitive impairments, ghrelin also increased retention in T-maze foot shock avoidance (Diano et al., 2006). In contrast with wild-type, knockout mice for the ghrelin hormone show deficits on the novel object recognition task, which the authors interpreted as a perturbation in the animal's ability to recall the objects that he was previously exposed to (Diano et al., 2006). This impairment was overcome after administering ghrelin subcutaneously. In line with this observation, subcutaneous injection of the ghrelin receptor agonist LY444711 also led to performance improvements in a spontaneous alternation plus-maze task (Diano et al., 2006). Additionaly, the oral administration of GSK894490A and CP-464709-18, two structurally related non-peptide ghrelin receptor agonists, also enhanced the performance in novel object recognition and water maze tests (Atcha et al., 2009). Conversely, GHS-R knockout mice displayed impairments in Morris water maze tests (Davis et al., 2011). After chronic food restriction mice show impairments on novel object recognition task, however, the ICV injection of ghrelin can rescue mice performance to control levels (Carlini et al., 2008). Of note, more recently it was shown that knockout mice for GHS-R1a were capable of habituating faster to a new environment, performed better in Morris water maze but displayed impairments in contextual fear conditioning (Albarran-Zeckler et al., 2012). This suggests that eventhough the cognitive enhancing characteristics of ghrelin are well ascertained, it still remains to be determined in detail in which contexts these enhancements occur.

The memory enhancing effect of ghrelin seems to be species-dependent. In opposition to the previous results, ICV injection of ghrelin in neonatal chicks reduced memory retention after a passive avoidance task (Carvajal et al., 2009). Also, the few studies that explored ghrelin's role on human cognition did not corroborate the findings in rodents. On one study, ghrelin had a negative effect on declarative memory on healthy elderly adults (Spitznagel et al., 2010). Additionaly, the increased performance in motor skill tasks normally observed after a night of sleep, was blocked by the administration of ghrelin, suggesting an impairment of sleep-related neuroplasticity (Dresler et al., 2010). These observations may shed light on possible difficulties in the direct application of the ghrelin peptide to disorders related with cognitive impairment.

Several molecular mechanisms have been suggested to explain the aforementioned cognitive effects of ghrelin. It is now known that ghrelin induces memory improvements by modulating hippocampal synaptic plasticity (Carlini et al., 2010; Diano et al., 2006), through several intracellular effectors (Carlini et al., 2010; Chen et al., 2011; Cuellar and Isokawa, 2011), by the regulation of modulatory neurotransmitter systems (Carlini et al., 2007), and by directly modulating hippocampal neurogenesis (Li et al., 2013a). An explanatory model for ghrelin-mediated memory improvements should seek to interrelate these various parts of the mechanism and further clarify how they improve memory *in vivo*.

Diano and co-workers showed that the peripheral administration of ghrelin significantly increased dendritic spine formation in CA1 region of the hippocampus (Diano et al., 2006). Moreover, in ghrelin knockout mice a decrease in hippocampal spine density was rescued after administration of ghrelin (Diano et al., 2006). *In vitro* treatment of organotypic slice cultures of hippocampus with ghrelin increases the number of F-actin clusters on CA1 (Berrout and Isokawa, 2012), and this increase is prevented by either the presence of a ghrelin receptor antagonist or the treatment of the cultures with ghrelin-free media for 22h, even after cells were previously treated for 1h with culture media with ghrelin (Berrout and Isokawa, 2012). These results show that the maintenance of ghrelin-induced increases in spine density may only persist in the presence of continuous activation of the ghrelin receptor. It is noteworthy that the authors did not observe a change in the density of ghrelin receptor after its activation, which suggests that the GHS-R1a may not be internalized during persistent activation (Berrout and Isokawa, 2012).

Long-term potentiation (LTP) is considered to be the molecular and cellular substrate for activity-dependent synaptic plasticity in the hippocampus, and a surrogate for spatial memory and memory retention (Bohme et al., 1993; Pavlides et al., 1991). Diano and co-workers showed that acute hippocampal slices treated with ghrelin for 30 min showed an increased excitatory postsynaptic potential (EPSP) slope after 10-Hz stimulation, but no change if using

theta burst stimulation (Diano et al., 2006). It should be noted however that a 10-Hz stimulation is not recognized as an electrical stimulation protocol for synaptic plasticity on the hippocampus (Albensi et al., 2007). I was subsequently shown the decline of high frequency stimulation (HFS)-induced LTP *in vivo* (180 min after LTP induction by HFS) is prevented by ghrelin injection and that this processess is dependent of MAP kinase being activated by the ghrelin receptor (Chen et al., 2011). Ghrelin only impacted the late-phase of LTP, which relies on the synthesis of new proteins, in contrast with early phase of LTP which depends on post-translational modifications of pre-existing proteins (Kelleher et al., 2004; Lynch et al., 2004). Using acute hippocampal slices, it was also shown that ghrelin administration into the CA1 region reduces the LTP induction threshold through HFS, and that this phenomenon is dependent on the activation of nitric oxide synthase (NOS) (Carlini et al., 2010).

The modulation of the serotonergic system may also be implicated in ghrelin's effect on memory. Increased memory retention elicited by ghrelin can be blocked by the previous intra-hippocampal administration of serotonin reuptake inhibitors (SSRI) (Carlini et al., 2007), hence, lower the levels of serotonin may be a requirement for ghrelin-mediated memory retention improvement (Carlini et al., 2002a; Carlini et al., 2004b). Indeed, in hippocampal slices acquired from animals trained in step down test, the administration of ghrelin to the CA1 region inhibited the secretion of serotonin (Ghersi et al., 2011). Linking this finding to the previous discoveries, it was proposed that the activation of GHS-R1a in the hippocampus could lead to increased intracellular free calcium concentrations, that contributed to the activation of NOS, leading to an inhibition of the release of serotonin release (Ghersi et al., 2011). In support of this theory are the observations that the increase in serotonin impairs LTP in various experimental paradigms (Corradetti et al., 1992; Kim et al., 2006; Kojima et al., 2003; Mori et al., 2001), and inhibitors of NOS can induce an increase in hippocampal serotonin levels (Wegener et al., 2000). Moreover, administration of a NOS inhibitor previously to step-down test inhibited ghrelin-mediated effects on memory retention (Carlini et al., 2010). Recently, it was shown that caloric restriction, which is already described to increase circulating levels of ghrelin, enhances fear extinction through a mechanism involving the increased reuptake of serotonin (Riddle et al., 2013).

The activation of PI3K is another component of the mechanism of ghrelin-mediated memory improvement. Ghrelin administration induces an increase in the excitability of postsynaptic granule cell as well as presynaptic transmitter release on the hippocampal dentated gyrus (Chen et al., 2011). This increased excitability is perturbed by the administration of AP5, a competitive antagonist of the N-methyl-D-aspartate receptor (NMDAR) (Chen et al., 2011). The application of ghrelin in the hippocampus also signaled the PI3K pathway, as shown by increase in phosphorylation of Akt Ser^{473}, and blockage of the synaptice response by administration of an

inhibitor of PI3K. Ultimatly, the PI3K inhibitor was able to prevent a ghrelin-mediated enhancement of spatial memory (Chen et al., 2011).

Another aspect of the ghrelin signaling mechanism is the activation of protein kinase A (PKA). Activation of PKA is observed after hippocampal organotypic slices are incubated with ghrelin for one hour in the presence (Cuellar and Isokawa, 2011). Ghrelin treatment increased the phosphorylation of CREB at Ser^{133}, which in turn was prevented by the GHS-R1a antagonist [$D\text{-}Lys^3$]-GHRP-6 or by a PKA inhibitor. Nevertheless AP5, an inhibitor of GluN2B-containing NMDAR, as well as by the endocannabinoids anadamide and 2 arachidonoylglycerol (2-AG) (agonists of the type 1 cannabinoid receptor (CB1R), a G_i protein-coupled receptor) were all able to inhibit the phosphorylation of CREB (Cuellar and Isokawa, 2011). After ghrelin treatment, it is observed an increase in phosphorylated GluN1, which is blockable by agonists of endocannabinoid receptors. These findings suggest that in the hippocampus, GHS-R1a activation enhances cAMP production, which by itself activates PKA; these events require that GHS-R1a be coupled to a G_s protein (reviewed in (Muccioli et al., 2007)). PKA activation may subsequently lead to enhancement of NMDA function and the observed increase in phospho-GluN1. It is of note that in order to clearly link the activation of PKA with GluN1 phosphorylation the phosphorylated residues in GluN1 should have been determined. Alternatively, the GHS-R1a may increase the production of cAMP through the amplification of dopamine-induced production of cAMP (Jiang et al., 2006). The aforementioned heterodimerization of the ghrelin with dopamine receptors may be relevant for the effect seen on learning, through the indirect modulating of signal transduction in dopamine systems. Indeed, the improvements on object location memory task observed after ghrelin administration are reversed by the D1 receptor-antagonist SFK83566 (Jacoby and Currie, 2011).

Hippocampal neurogenesis may also be implicated in ghrelin-mediated memory enhancement. Ghrelin peptide knockout mice show reduced numbers of progenitor cells in the subgranular zone of the hippocampus (Li et al., 2013a). This decrease can be recovered through the intraperitonial administration of exogenous ghrelin. Furthermore, ghrelin also corrected impairments of memory performance in Y-maze task and novel object recognition, observed in the knockout mice for ghrelin peptide (Li et al., 2013a) and a 4-week treatment with ghrelin also improved learning and memory mediated by the hippocampus, as examined through Y-maze and novel object recognition (Li et al., 2013b).

It should be noted that other brain regions apart from the hippocampus might be involved in this effect by ghrelin. Carlini showed that administration of ghrelin to the amygdala and dorsal raphe nucleus also improved memory retention, although the hippocampus was the region most responsive to this effect (Carlini et al., 2004a).

1.4 THE GLUTAMATERGIC SYNAPSE

Since the main effects of ghrelin explored in this thesis are related with memory and learning, and with the glutamatergic synapse, in the following section I will introduce the glutamatergic synapse and its role on memory and learning.

In order for neurons to create circuits capable of recieving, processing and outputing information, it is crucial that they comunicate with one another. The points of functional contact between neurons are known as synapses. The synapses can be chemical or electrical in nature, with essentially different characteristics. The chemical synapses represent the majority of synapses found on the brain. The structure of the chemical synapse consists of a pre-synaptic terminal, where action potentials arrive and eventually lead to the release of neurotransmitter molecules on the terminal, a synaptic cleft, through which the neurotransmitters are dispersed, and a post-synaptic terminal, on the surface of which can be found receptors for the specific neurotransmitters released by the presynaptic terminal, that once activated can alter the membrane potential. These synapses can lead to either the hyperpolarization (inhibitory post-synaptic potentials or IPSP) or depolarization (excitatory post-synaptic potentials or EPSP) of the membrane potential (reviewed in (Byrne and Roberts, 2009)).

The vast majority of neurons on the human brain use glutamate as a neurotransmitter. These synapses are involved in processing of sensory information, emotions and cognition among other brain functions. Glutamate is an anionic aminoacid, and its receptors induce depolarization of the post-synaptic membrane. The glutamatergic synapse has the particularity of possessing a thick protein-rich region on the post-synaptic terminal, known as the post-synaptic density or PSD (reviewed in (Byrne and Roberts, 2009)).

The PSD is ~50 nm thick, and apart from possessing the post-synaptic glutamate receptors, contains a large array of protein involved on scaffolding, trafficking and regulation of these and other receptors (Brady et al., 2012). The PSD can be found at the end of dendritic spines and its size correlates with the size of the spines and the amount of glutamate receptors (Sheng and Kim, 2011). The protein repertoire of the PSD includes most abundantly Ca^{2+}/calmodulin-dependent protein kinases (CaMK) and proteins of the membrane-associated guanylate kinase family (MAGUK) like postsynaptic density protein 95 and 93 (PSD-95 and PSD-93, respectively). PSD-95, and homologous proteins, bind to and stabilize signaling and membrane proteins in the PSD (Kim and Sheng, 2004): for instance, PSD-95 is capable of binding the carboxy-terminal of NMDA receptor subunit NR2, through its PDZ domain, and also signaling protein such as nitric oxide synthase or kalirin-7, thus coupling functionally coupling NMDA receptors with these proteins. PSD-95 also participates on the synaptic recruitment and

stabilization of AMPA receptors. The vast protein repertoire of the PSD is briefly summarized on figure 5.

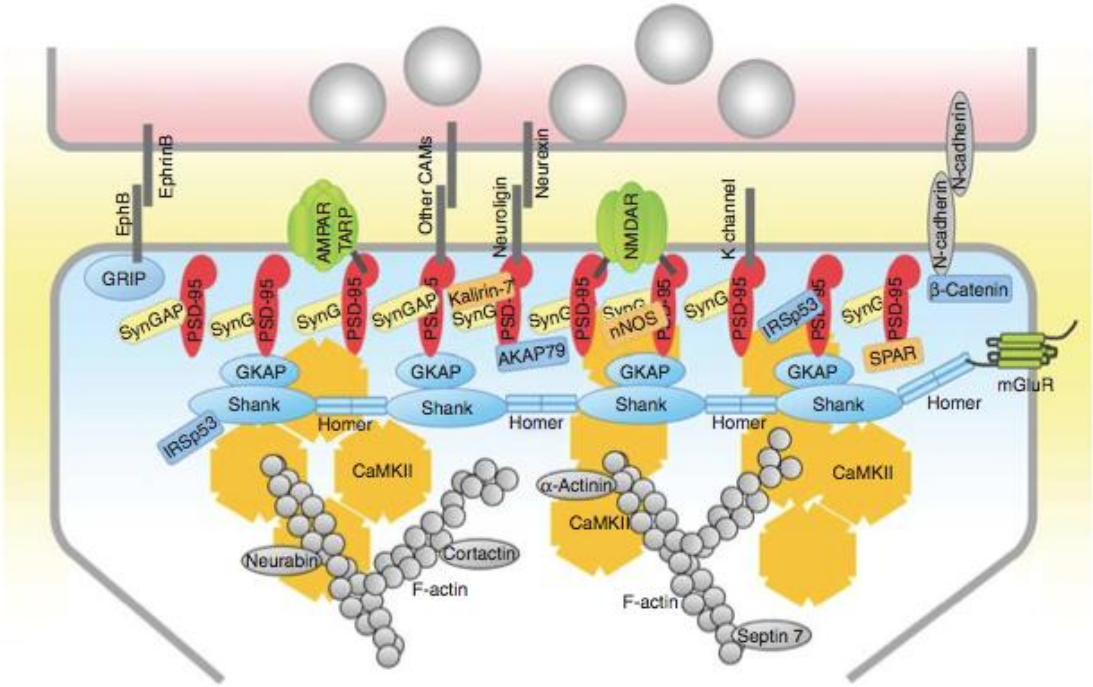


Figure 5 – Molecular organization of the PSD of excitatory synapses. Diagram of the most abundant proteins found on the PSD and their interactions. The number of proteins here represented correlate roughly with their abundance in forebrain PSDs. Each CaMKII shape represents a dodecamer. GRIP, glutamate receptor interacting protein-1; SynGAP, Synaptic RasGTPase-interacting protein 1; EphB, Ephrin receptor-B; AKAP97, A-kinase anchoring protein 97; mGluR, metabotropic glutamate receptor (adapted from (Sheng and Kim, 2011)).

1.4.1 Glutamate Receptors

Glutamate receptors can be divided in two main groups: Ionotropic receptors, cation permeable channels regulated by glutamate binding; or metabotropic channels, G-protein coupled receptors activatable by the binding of glutamate (Brady et al., 2012). Ionotropic glutamate receptors (iGluRs) produce fast excitatory synaptic transmission. They are capable of very rapidly decoding chemical signals into electrical signals, through their fast opening induced by the binding of glutamate (Smart and Paoletti, 2012). Membrane depolarization can be achieved by the opening of these receptors and they are primarily found on post-synaptic membranes. Four major families, constituted by 18 genes, compose the mammalian iGluRs: the AMPA, kainate,

NMDA, and delta receptors (Hollmann et al., 1989; Nakanishi and Masu, 1994; Seeburg, 1993). The members of the AMPA and NMDA family are the main receptors responsible for excitatory postsynaptic currents. Each family is further divided in four AMPA receptor genes (GluA1-4); five kainate receptors genes (GluK1-5); seven NMDA receptor genes (GluN1; GluN2A-D; GluN3A-B, also known as NR1; NR2A-D; NR3A-B), and two δ-subunits (GluD1-2, also known as δ1 and δ2). AMPA receptors subunits form homo- and heteromers. NMDA receptors are always found as heteromers associating two GluN1 subunits with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits (Cull-Candy and Leszkiewicz, 2004; Paoletti, 2011; Ulbrich and Isacoff, 2007). The subunit composition confers different singaling and electrical properties to the iGluRs. Different neuron populations tend to express a specific repertoire of subunits that may varie with development and activity (Smart and Paoletti, 2012).

The EPSCs are typically the result of the activation of AMPAR and NMDAR receptors. Upon binding to AMPARs and NMDARs, glutamate generates a two-component exciatory current: activation of AMPAR generates a component with rapid onset and decay, and NMDAR receptor activation produces a current with slower kinetics taking around several hundred milliseconds. The long time course of NDMA receptor activation enables temporal and spatial summation of multiple inputs, which is an important propertie for circuit level computations (Brady et al., 2012). NMDARs subunits are Ca^{2+} and Na^{+}-permeable and require glycine as a co-agonist. They are blocked by Mg^{2+}, which is overcome by depolarization. The NMDARs only conduct currents with simultaneous binding of glutamate and membrane depolarization, so pre- and postsynaptic neurons need simultaneous activation to open NDMARs (Brady et al., 2012).

1.4.2 AMPA receptors

AMPARs are impermeable to Ca^{2+} and mostly Na^{+}-permeable, and have fast-kinetics (Jonas, 2000). The receptor subunit composition and post-translational modifications determine its kinetic characteristics. The GluR1-GluR4 subunits share 68-74% amino acid sequence identity (Collingridge et al., 2004) and have four hydrophobic domains: the transmembrane domains TM1, TM3 and TM4 and the cytoplasmic M2 domain (Madden, 2002). The length of C-terminal cytoplasmic tails varies among AMPA receptor subunits and is regulated by alternative splicing (Shepherd and Huganir, 2007). Regarding subunit expression, AMPARs on the rat hippocampus are usually composed of GluA1 and GluA2 subunits or GluA2 and GluA3 subunits, GluA4 being mainly expressed during early development (Wenthold et al., 1996). Each subunit has specific protein interaction motifs on its cytoplasmic tail, and intracellular protein interacting with these motifs are essential for controlling the trafficking and stabilization of AMPA receptors at the synapses (Santos et al., 2009).

Phosphorylation is a crucial regulating modification of AMPAR function (Carvalho et al., 2000). Receptor trafficking into and out of the synapses and the physiological properties of their channels are modulated by phosphorylation. For instance, on GluR1 cytoplasmic tail, Serine^{831} and/or Serine^{845} phosphorylation are crucially involved in processes of synaptic accumulation (Esteban et al., 2003) and kinetics of the of AMPA receptors (Banke et al., 2000). Other modifications occurring on AMPAR include palmitoylation (Lin et al., 2009), glycosylation (Kanno et al., 2010) and ubiquitination (Fu et al., 2011) of GluA1.

Endocytosis and exocytosis of the AMPA receptors regulate their presence at the synaptic membrane. Increased exocytosis is crucial for synaptic potentiation while receptor endocytosis leads to synaptic depression (Kessels and Malinow, 2009; Shepherd and Huganir, 2007). Upon LTP induction, AMPA receptors are inserted at perisynaptic sites, by the activation of PKA and its phosphorylation of Serine^{845} (Figure 6), are stabilized by actin polymerization and move to the synapse (Yang et al., 2008b). The mechanism of AMPAR exocytosis is specific to each subunit, with exocytosis of GluR2 being fast and constitutive, and GluR1 exocytosis inducible but slow (Passafaro et al., 2001). The AMPAR diffusion from extrasynaptic to synaptic sites depends on the interaction of PSD-95 and stargazin, a transmembrane AMPA receptor regulatory protein (TARP) (Bats et al., 2007). TARPs are responsible for the regulation of AMPAR trafficking and channel kinetics (Kato et al., 2010) stabilizing AMPAR in the synaptic membrane through direct interaction with PSD-95 (Bats et al., 2007). LTP induction leads to the phosphorylation of stargazin by CaMKII and PKC, which enable its interaction with PSD-95 and thus the stabilization of AMPA receptors at the PSD (Tomita et al., 2005). Stargazin also regulates AMPAR kinetics by inhibiting glutamate-induced AMPAR deactivation and desensitization, and enhancing channel condutance (Nicoll et al., 2006; Priel et al., 2005; Tomita et al., 2005).

AMPARs internalized by clathrin-dependent endocytosis (Beattie et al., 2000; Man et al., 2000), and depending on the interation between GluR2 and AP2, a clathrin adaptor protein (Lee et al., 2002). AMPAR internalization can be caused by various stimuli including activation of NMDARs (Ehlers, 2000), AMPARs (Lin et al., 2000), mGluRs (Xiao et al., 2001) or insulin receptors (Lin et al., 2000). AMPAR may be sorted for recycling or for degradative pathways depending on the stimulus inducing endocytosis (Ehlers, 2000; Lin et al., 2000). A specialized compartment located in lateral domains of the spine, the endocytic zone, is dedicated to AMPA receptor internalization (Blanpied et al., 2002).

In the absence of neuronal activity, AMPARs are constitutively cycled between the synaptic membrane and the cytosol, where they can be degraded or sorted for reinsertion in the membrane (Ehlers, 2000; Passafaro et al., 2001), figure 6.

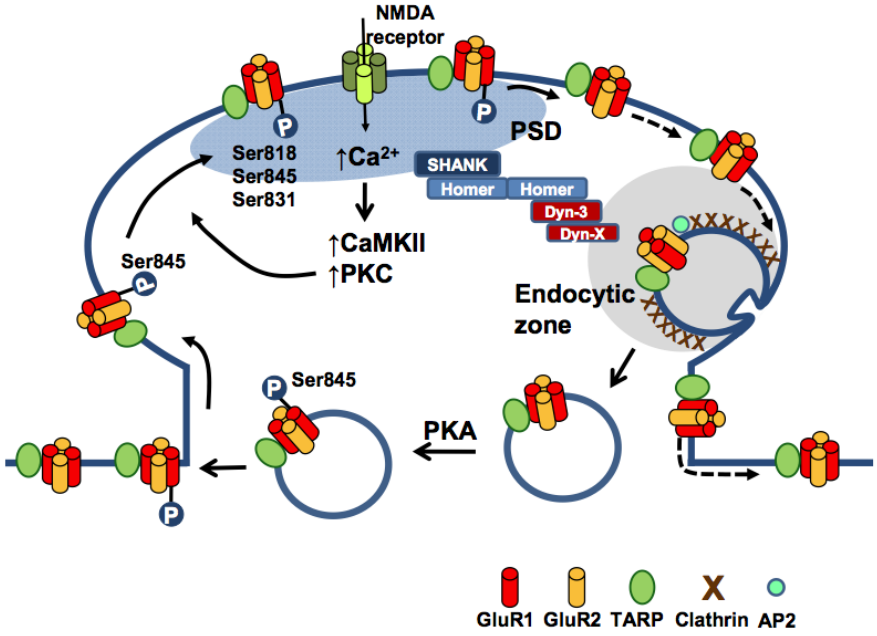


Figure 6 – Regulation of intracellular traffick of GluR1-containing AMPA receptors. On the PSD, the AMPA receptors have a limited mobility, in contrast with the non-synaptic receptors that can freely diffuse on the membrane. The internalized receptors at the endocytic zone by a clathrin-mediated process may afterwards by recycled. The positioning of the endocytic zone is determined by the association of dynamin 3 (Dyn-3) with Homer and SHANK. Apapted from (Santos et al., 2009)

1.4.3 Learning and memory

Learning is a crucial aspect of behavior. Complex learning processes lie at the core the organisms ability to purposefully acquire and process sensory information, distinguish favorable stimuli from harmful ones and form new memories. In our current understanding, the cellular basis of such information acquisition and storage processes lies at the synaptic level, and is mediated by a collection of processes referred to as "synaptic plasticity". There are two main electrophysiological phenomena thought to represent memory at the synaptic level: long-term potentiation (LTP) and long-term depression (LTD) of synaptic excitability. LTP is mainly induced through the high-frequency stimulation (e.g. 100 Hz) of the synapse, which afterwards may relay impulses with higher efficiency for an extended period of time. On the other hand, a prolonged low-frequency stimulation (1 Hz) may lead to the prolongued decrease of the synapses excitability, which consists on LTP (reviewed in (Brady et al., 2012)). Regarding LTP induction, both pre- and postsynaptic neurons need to be simultaneously activated in order

NMDAR Mg^{2+} block to be fully relieved (discussed in the section "Glutamate Receptors"), figure 7. This ensures the influx of calcium through NMDARs, which activates intracellular signaling molecules that alter synaptic efficacy (Luscher and Malenka, 2012). In contrast with this mechanism, LTD is induced by repeated low frequency activation of the presynaptic neurons, without postsynaptic activity. In this case a lower amount of calcium enters the cell (Bloodgood and Sabatini, 2007, 2009; Sabatini et al., 2002) triggering LTD induction. LTP and LTD is induced at a cell and synaptic-specific level (Luscher and Malenka, 2012).

Activation and trafficking of glutamate receptors mediates the LTP and LTD phenomena (Brady et al., 2012). During LTP, heteromeric GluA1/GluA2 receptors are the main subtype of AMPARs inserted into the synapse (Adesnik and Nicoll, 2007). The exchange of GluA2-containing AMPARs in the synapse for GluA2-lacking AMPARs takes place in other forms of synaptic plasticity (Liu and Zukin, 2007), the latter having higher conductance. TARP family members interact with all AMPAR subunits and control their insertion into the plasmatic membrane and redistribution to the synaptic membrane (Rouach et al., 2005).

There are strong evidences that during LTP the opening of NMDARs increases the intracellular free calcium concentration to optimal levels for the activation of CaMKII (Lisman et al., 2002). This results in mainy synaptic proteins to be phosphorylated including AMPARs (Derkach et al., 1999), which increases their conductance and contributes to its insertion in the synaptic membrane (Ehlers, 2000). Additionally, more protein kinases, including PKA, PKC, MAPK and tyrosine kinases, take part on the phenomenon of LTP (Bliss and Collingridge, 1993; Nicoll and Malenka, 1999; Sweatt, 2004). The signaling of LTD depends, on the other hand, on the activation of calcium/calmodulin-dependent protein phosphatase, calcineurin, and protein phosphatase 1 (PP1) (Lisman, 1998). The differential activation of these calcium-sensing proteins is explained by the higher affinity of calcineurin for calcium/calmodulin than CaMKII (Mulkey et al., 1994; Mulkey et al., 1993). The processes of LTP and LTD also involved changes in the ultrastructure of the synaptic and the dendritic spine: while LTP is accompanied by the appearance of new spines within minutes after the induction protocol (Toni et al., 1999) and the enlargement of the PSD (Toni et al., 2001), LTD involves the shrinkage and disappearance of dendritic spines (Kasai et al., 2010). The LTP induced synaptic strength changes are maintained by potein synthesis-dependent processes. PKA, CaMKIV, protein kinase M-ζ, and extracellular signal-regulated kinase (ERK), among other signaling molecules, signal protein synthesis either locally or by nuclear transcription (Sacktor, 2008).

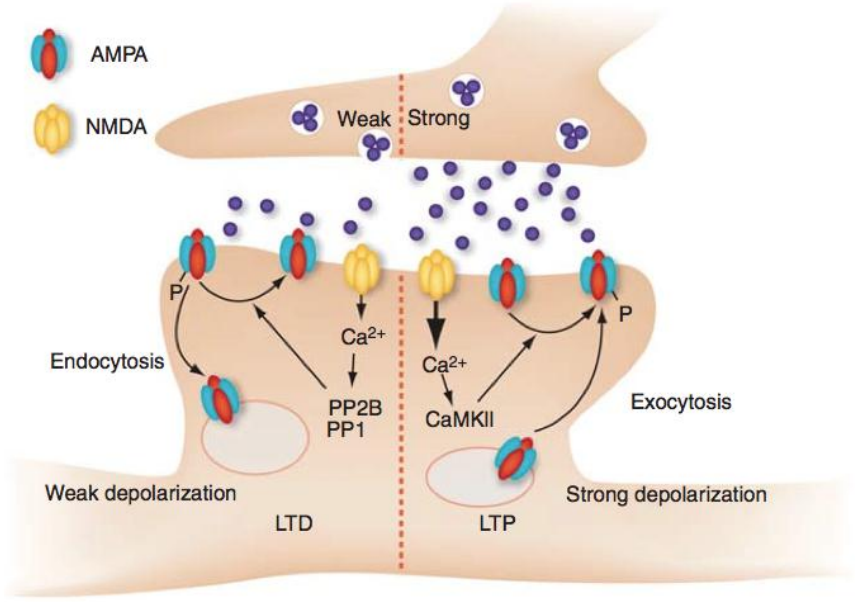


Figure 7 – Postsynaptic expression mechanisms of LTP and LTD. A weak presynaptic activity results in modest depolarization and calcium influx through NMDA receptors, leading to the activation of various phosphatases and subsequent dephosphorylation of AMPA receptors, which promotes their endocytosis. Strong depolarization induced by strong presynaptic and postsynaptic activity triggers LTP partially due to the activation of CaMKII, phosphorylation of glutamate receptors, and their exocytosis. Adapted from (Luscher and Malenka, 2012).

1.5 RECENT FINDINGS REGARDING EFFECTS OF GHS-R1A IN THE GLUTAMATERGIC SYNAPSE

The recent work from Ana Luísa's lab has shown that both ghrelin and the ghrelin receptor agonist MK-0677, through the activation of GHS-R1a, can induce the synaptic incorporation of GluA1 in rat hippocampal neuron cultures and in organotypical slices. This process was shown to be dependent on activity and NMDAR activation. MK-0677 infusion of organotypical slices was shown to enhance LTP expression. This argues in favor of GHS-R1a activation facilitating the canonical NMDAR-dependent LTP. Application of MK-0677 on neuron cell cultures for 1 or 20h before chemical LTP (cLTP) protocol was also able to increase GluA1 content on the membrane surface and synapses. The PKC pathway was upregulated by MK-0677, with a significant increase after 5h of treatment, and activation of the PI3K pathway after 30 min. GluA1 phosphorylation at Serine^{831} and Serine^{845} were also increased and 20h of MK-0677

induced stargazin phosphorylation at Serine$^{239/240}$ (Ribeiro et al., 2014). Figure 8 summarizes the proposed mechanism.

Furthermore it was shown that GHS-R1a expression increases consistently during development in cultured hippocampal neurons and the knockdown of the receptor expression impacts arborization complexity in young hippocampal neurons, suggesting that GHS-R1a is important on the process of dendritic arborization during development. Dendritic filopodia formation was increased by MK-0677 treatment, with a simultaneous decrease in some types of more mature spines. Knockdown of GHS-R1a produced opposite effects, along with decrease in expression of synaptic proteins (PSD-95 and Vglut1). In more mature neurons, GHS-R1a activation enhances the expression of synaptic proteins, while pharmacological inhibition of the constitutive activity of GHS-R1a decreases clustering of synaptic PSD-95 and Vglut1. F-actin dendritic clustering was increased by MK-0677 treatment, suggesting spine enlargment, maturation, formation, or all these processes. Agonist-induced and constitutive activity of the receptor thus seems to be important for spine formation and/or maturation in mature neurons. Activation of GHS-R1a also increased surface expression of GluA1 in hippocampal slices; this may be part of a ghrelin mediated priming of AMPARs for synaptic incorporation, a mechanism that may be integrated on the aforementioned LTP-enhacing effects of GHS-R1a activation (Fiona et al., unpublished work).

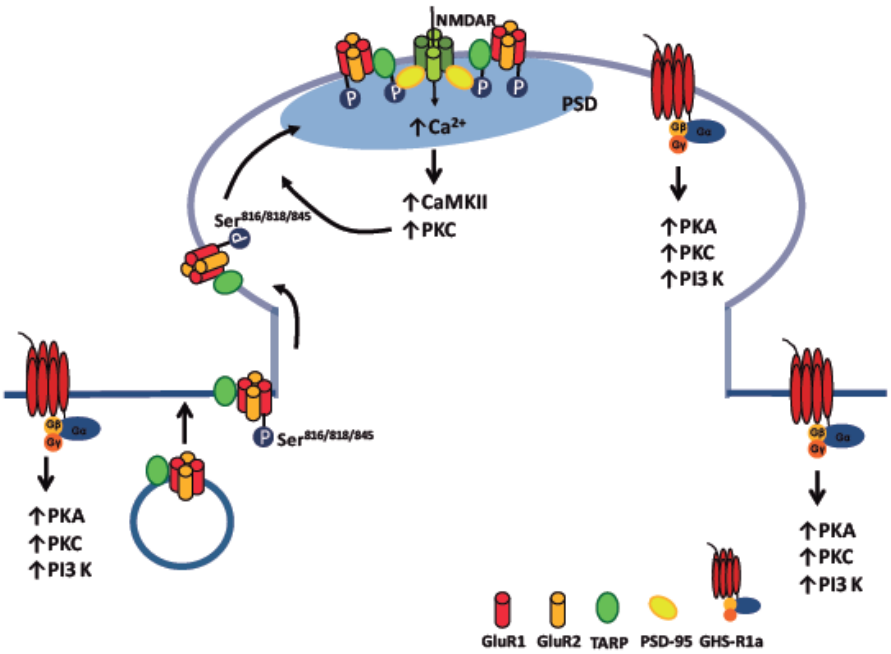


Figure 8 - Model of GHS-R1a-mediate enhacement of synaptic strength. The GHS-R1a is localized in proximal postsynaptic position in the glutamatergic synapse, being thus capable of directly influencing glutamatergic post-synaptic signaling processes. GHS-R1a activation triggers PKA and PKC signaling pathways that phosphorylate GluA1 C-terminus in the residues Ser^{845}, Ser^{816} and Ser^{818} leading to GluA1 trafficking to the plasmatic membrane. NMDARs activation during LTP induction leads to the activation of CaMKII, which induces GluA1 synaptic insertion. CaMKII also phosphorylates TARPs contributing to the binding of GluA1 to PSD-95 and thus retaining the receptors on the synapse. These biochemical alterations enhance the GluA1-containing AMPARs synaptic upon LTP induction. Adapted from (Santos et al., 2009)

2 OBJECTIVES

In this thesis work we had two main objectives. In order to build a bridge between the previous observation of the increase in AMPA receptor synaptic incorporation and the memory enhancing properties of ghrelin, we aimed at understanding whether the abrupt decreases of ghrelin levels, which are ubiquitous during the daily oscillation of plasmatic ghrelin levels, had an impact on the post-synaptic density molecular composition in the hippocampus, and if so, how is the glutamatergic synapse molecular composition altered. The other objective was the elucidation of the effects of ghrelin on anxiety through testing the hypothesis that the period of time between ghrelin administration and start of the behavioral tests affects anxiety.

Chapter 2 – Material and Methods

3 MATERIALS AND METHODS

3.1 ANIMALS

For the behavioral experiments, male C56BL/6 mice from Charles River were used. The animals were kept in the Animal Facility of the Faculty of Medicine of the University of Coimbra with access to food and water *ad libitum.* The animals were kept in temperature and humidity controlled conditions under a 12h dark-light cycle (light period 6h00-18h00). For organotypic slice cultures, Wistar rats of postnatal day 7 of both sexes were used from the in-house colony. Behavioral testing was performed in accordance with the guidelines from the animal use committees (ORBEA) at Center for Neuroscience and Cell Biology, University of Coimbra.

3.2 REAGENTS

Rat ghrelin was purchased from Tocris Bioscience (Bristol, UK). The GHS-R1a agonist MK-0677 was purchased from Axon Medchem (Groningen, Netherlands). They were both reconstituted in 1x PBS, at pH 7.4. All other reagents were purchased from Sigma-Aldrich (Sintra, Portugal) or from Fisher Scientific (Loures, Portugal) unless specified otherwise.

3.3 ANTIBODIES

Primary Antibodies	**Dilution**	**Source**
Beta-Tubulin	1:200.000	Sigma-Aldrich (Sintra, Portugal)
PDS-95	1:2000	Cell Signalling Technologies (Danvers, USA)
GluA1	1:1000	Millipore (Madrid, Spain)
Synaptophysin	1:100.000	Abcam (Cambridge, UK)
NR-1	1:500	Invitrogen

Secundary Antibodies	Dilution	Source
Horse radish peroxidase conjugated donkey anti-rabbit	1:20.000	Jackson Laboratories (Baltimore Pike, USA)
Horse radish peroxidase conjugated donkey anti-mouse	1:20.000	Jackson Laboratories (Baltimore Pike, USA)

3.4 HIPPOCAMPAL ORGANOTYPIC SLICE CULTURES

Hippocampal slices cultures were performed as previously described (Gahwiler et al., 1997). After dissection of the hippocampi in ice-cold carbogenated (5% CO_2/95% O_2) dissection solution (in mM: 10 glucose, 4 KCl, 24 $NaHCO_3$, 234 sucrose, 0,5 $MgCl_2$, 0,7 $CaCl_2.2H_2O$, 0,03 phenol red, at pH 7,4), 400 μm transverse slices were prepared using a tissue slicer. Both hippocampi from the same animal were mixed. Slices were transferred to slice culture inserts (Millipore, Madrid, Spain) and cultured in culture medium [Minimum Essential Media (MEM; GIBCO, Invitrogen, Barcelone, Spain), 1mM glutamine, 1 mM $CaCl_2$, 2 $MgSO_4$, 1 mg/l insulin, 0,0012% (w/v) ascorbic acid, 30 mM HEPES, 13 mM glucose, 5,2 mM $NaHCO_3$, at pH 7,25-7,26]. 7 slices were used per insert. Cultures were maintained in a humidified incubator of 5% CO_2/95% air at 35°C, and the culture medium was replaced every other day. Slices were used after 3 days in vitro. Each experimental condition used 42 slices.

3.5 POST-SYNAPTIC DENSITY PURIFICATION

The protocol for post-synaptic density purification was adapted from (Peca et al., 2011). This protocol uses solution A (4mM HEPES, 0.32M sucrose), B (4mM HEPES, no sucrose) and C (50mM HEPES, 2mM EDTA). All solutions contain 1 μg/mL chymostatin, 1 μg/mL leupeptin, 1 μg/mL antipain, 1 μg/mL pepstatin (CLAP), 0.1 mM of sodium orthovanadate and 50 mM of sodium fluoride. Per condition, slices were transferred to 3mL of cold solution A and homogenized in a motor driven glass-teflon homogenizer at ±900 rpm, 50 strokes, at 4°C. The samples were then stored at -80°C before further processing. Beginning on the following days, samples were thawed and centrifuged at 700g, 4°C for 15 min to remove the nuclear fraction. This step was performed twice. At this point a sample from each condition was collected, corresponding to total lysate (300 μL of sample, 200 μL of solution A, 43 μL of SDS 20% and 90 μL of Urea 9M). The remainder was centrifuged at 18.000g, 4°C for 15 min to yield the crude synaptosomal pellet. It was then resuspended in 1 mL solution A and centrifuged at 18.000g for 15 min to yield the washed crude synaptosomal fraction. The resulting pellet was lysed by hyposmotic shock by adding 12 mL of cold solution B and applying 8 strokes on a homogenizer. The solution was transferred to falcon tubes and mixed in a shaker, 4ºC for 1h. Afterwards, using Beckman

Ultra-Clear Centrifuge Tubes (3445059), the lysates were centrifuged at 25.000g for 20 min. The pellet was resuspended in 500 μL of solution A, then diluted to 0.32M sucrose by adding 2.5 volumes of solution B, and finally pelleted by centrifugation at 150.000g for 30min. To avoid the collapse of the centrifuge tubes, an overlay of solution B without inhibitors was applied. The pellet was resuspended in 500 μL solution C and a sample from each condition was collected, corresponding to synaptossomal plasma membrane (SPM) (100 μL sample, 50 μL solution C, 30 μL SDS 20%). At this point the samples could be stored at -80°C if the remainder of the protocol could not be immediately performed. Afterwards, solution C was added up to 1 mL, and Triton X-100 was added to 0.5%. The solution was then mixed and rotated for 15 min at 4°C. It was then centrifuged at 32.000g for 20 min. The pellet was resuspended in 1 mL of cold solution C and 0.5% (v/v) of Triton X-100 was added, and the solution was incubated for 15 min at 4°C. It was then centrifuged at 200.000g for 20 min. The pellet corresponded to the purified postsynaptic density fraction and was resuspended in 200 μL solution C, to which 29 μL SDS 20% and 90 μL Urea 9M was added. The obtained samples were all stored at -20°C. To validate the efficacy of the purification, western blots for PSD-95, β-tubulin and Synaptophysin were performed. A successful purification displayed a characteristic increase in the levels of PSD-95 and decrease on the level of synpatophysin on the PSD fraction in comparison with the remainder fractions (an example blot for quality control is shown in the supplementary results). This protocol was previously validated by transmission electron microscopy (Cohen et al., 1977).

3.6 WESTERN BLOTTING

Protein samples were resolved by SDS-PAGE in 10% polyacrylamide gels, of 1.5 mm. The 10% running gel was composed of 3.5 mL of H_2O, 2.875 mL of 1.5M Tris at pH 8.8, 2.2 mL of 40% Acrylamide, 87.5 μL of 20% SDS, 120 μL of 10% AMPS and 17.5 μL of TEMED. The 4% stacking gel was composed of 2.285 mL of H_2O, 0.95 mL of 0.625M Tris at pH 6.5, 375 μL of 40% Acrylamide, 37.5 μL of 20% SDS, 56.25 μL of 10% AMPS and 7.5 μL of TEMED. Before loading, samples were mixed with Laemmli sample buffer (Biorad) and incubated at 91°C for 5 min. The samples were loaded at 5 μg. For western blot analysis, proteins were transferred onto a PVDF membrane (GE Healthcare) by electroblotting (constant voltage 100 V, for 2h). The membranes were briefly washed in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% (v/v) Tween-20 (TBS-T) and then blocked on 5% (w/v) low-fat milk in TBS-T for 45 min with shaking. Membranes were probed with primary antibodies overnight at 4°C. Afterwards they were washed 3 times with TBS-T (15 min each wash) and then probed with secondary antibodies for 2h at room temperature. Afterwards the membranes were again washed 3 times with TBS-T (15 min each wash) and incubated with ECL-Plus (Thermo Scientific) for 5 min. The membranes were then scanned using a Storm 860 scanner (GE Healthcare, Carnaxide, Portugal) and quantified using Fiji Western Blot quantification plugin.

3.7 BEHAVIORAL TESTS

For all behavioral tests, the animals were transferred from the animal house at ~11:00 and given 1 hour to acclimatize to the behavioral room; tests were conducted from ~12:00 until ~17:30 and afterwards the animals were returned to the animal house. Drug and saline administration were performed by intra-peritoneal injections. Each animal was weighted to determine injection bolus. The ghrelin injected was administered at 2 μ g/g of animal weight, based on prior studies (Lutter et al., 2008). The amount of MK-0677 injected was 0.5 μ g/g of animal weight based on the recent work by Meyer and colleagues (Meyer et al., 2013). Drugs were reconstituted 2-3 days before the behavioral test and individual aliquots for each animal were prepared and stored at -20ºC. At the same instance, aliquots of the vehicles (PBS), to inject in the control animals, were also prepared and frozen. On all tests the room temperature was maintained at 23°C ±1°C. The scheme and intensity of illumination depended on each test.

3.7.1 Open-field test

We used a commercial open-field arena (Panlab, Barcelona, Spain) with infrared tracking of the animal position. The protocol for this behavioral paradigm was based on (Gerfen, 2006). This test was conducted on a first cohort of mice (~9 weeks old, 20 animals) during 21-25 of October of 2013, and on a second cohort of mice (~12 weeks old, 20 animals) during 24-28 of February of 2014. The animals treated with MK-0677 were tested during 31 of May and 1 of June of 2014 (~16 weeks old, 14 animals). 4-5 animals were tested each day for ghrelin experiments, and 7 animals were tested per day for MK-0667 experiments. Indirect and homogenous illumination of the room was provided from white LED lamps and the surface of the open-field arena was maintained at 10 lx. The open-field test was run for 1h. During the periods between drug administration and testing, the animals were transferred to a transport cage, without chow or water. After the test the animals were transferred to a different transport cage, with chow and water, until they were transferred back to their home cage. Between animal tests the arena was cleaned with 75% ethanol. Time on the center area of the arena and distance travelled were evaluated. This data was obtained by analyzing animal behavior with Actitrack software. Files created by this software were used to create heatmaps of the animals location on the arena, with the help of custom made Matlab scripts. The runs for the second cohort of animals were also filmed to evaluate grooming and rearing activity. The videos were manually analyzed with the help of a costum made Matlab application.

3.7.2 Elevated Plus Maze

This maze was built in-house according to the dimensions and materials specified in (Gerfen, 2006). The maze was built from semi-opaque reflecting white acrylic (0.5 cm thickness) and supported above ground with PVC tubes. A rim of 0.5 cm of height was built around the open arms to prevent excessive falling of the animals (Matsuo et al., 2010). A scheme of the maze and a photography of the built maze is shown in figure 9.

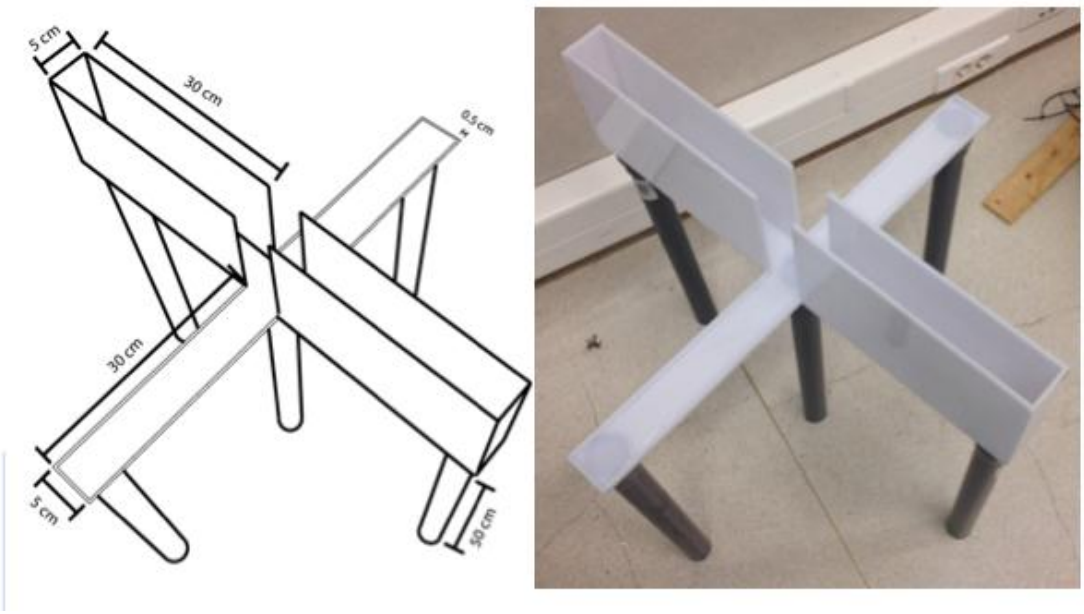


Figure 9 - Elevated Plus Maze scheme and the one built in the lab

The protocol for this behavioral paradigm was based on (Gerfen, 2006; Matsuo et al., 2010). This test was run during the weekend of 15-16 of March of 2014 (19 animals, ~9 weeks old). The illumination was provided from white LED lamps positioned over the maze, with the surface of the maze illuminated at ~100 lx. The duration of the test was 10 minutes and was started by introducing the mouse to the center part of the maze towards an open arm. Four experimental groups were established similarly to the open-field test. Mice were filmed with a camera positioned above the maze. The videos were analyzed to obtain the time each animal spent on the closed or open arms and the number of times they enter in each type of arm. This analysis was done with the help of an costum made application using Matlab. This application assigned key strokes or button-push to the position in which the animal was on the maze (center, closed arms or open arms) and reported of where the animal was at each particular moment during the run. The user interface of the application is shown on figure 10. The source code for this application can be found on the supplementary methods section.

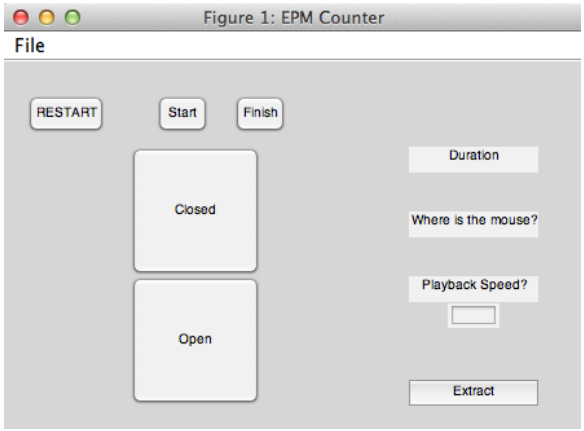


Figure 9 - Elevated Plus Maze position counter application

Our control experiments were compared with the literature (Matsuo et al., 2010) to validate our arena and coding methodology.

3.7.3 Dark-Light Box

This maze was built in-house according to the dimensions and materials specified on (Gerfen, 2006; Matsuo et al., 2010). The maze was made from opaque non-reflecting white PVC (1 cm thickness). This maze consisted of an open-field arena divided into a light and dark part, with a hole in between the two parts to let the animal transition from one area to the other, and a hole on top of the dark box so the animal could be positioned at the onset of the test. Two square pieces of PVC were provided to enable the closure of both holes. The piece dividing both compartments is removable, which facilitates cleaning. A scheme of the maze and a photography of the maze are shown in figure 11

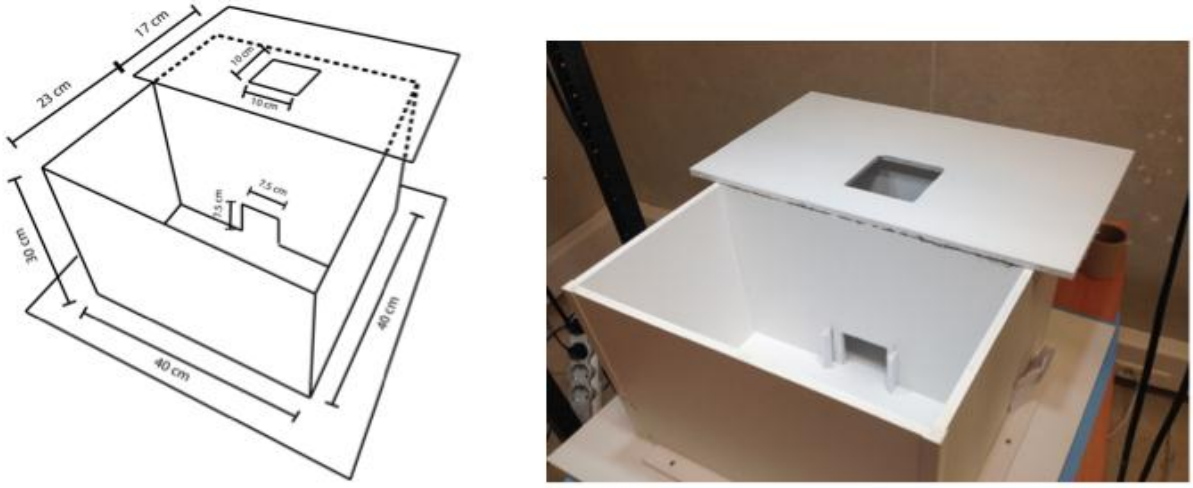


Figure 11 – Dark-Light Box scheme and the one built in the lab

The protocol for this behavioral paradigm was based on (Gerfen, 2006; Matsuo et al., 2010). This test ran during the weekend of 24-25 of May of 2014 (19 animals, ~15 weeks old). The illumination was provided from white LED lamps positioned over the maze, with the surface of the light part illuminated at 400 lx. The test ran for 10 minutes and began by introducing the animal on the dark part of the maze. Four experimental groups were established following the same intervals between drug administration and beginning of the test as the open-field test. Mice were filmed with a camera positioned over the maze. The videos were manually analyzed to obtain the time animals spend on the light versus the dark part of the maze, the number of times they transition to each part and latency to first enter the light. This analysis was done with the help of an in-house built application using Matlab software. This application assigned keystrokes or button-push to the position the animal was on the maze (light or dark part) and reported where the animal was at what particular moment during the run. The user interface of the application is shown in figure 12. The code for this application can be found on supplementary methods.

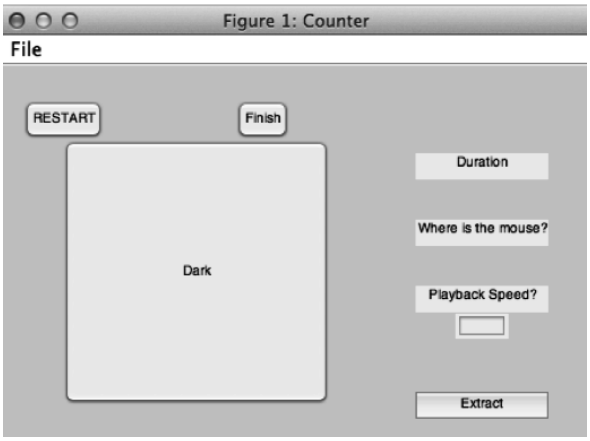


Figure 10 – Dark light Box position counter application

Our control experiments were compared with the literature (Matsuo et al., 2010) to validate our arena and coding methodology.

3.7.4 Statistical Analysis

For the western blots quantification band intensity was calibrated to each respective loading control and band intensities were normalized across experiments. Because PSD purification procedures where performed in side by side for each control-experimental pair, Student´s paired t-student test were used to compare averages between groups. Two-way ANOVA with Bonferroni posttest and unpaired t-student test were used for behavioral results. For biochemistry and behavior, Grubbs test was used to determine the presence of outliers. Statistical analysis was performed with Matlab and Prism (Graphpad).

3.8 SUPPLEMENTARY METHODS

The applications developed for this thesis were compiled as standalone apps to facilitate usage and portability. A grooming and rearing counting application was also made, by costumizing the applications for EPM and DLB counting.

3.8.1 Sourcecode for EPM counter application

```
function epmcounter
%% Setting the common application variables
f = figure('Visible','off','Position',[360,500,450,285]);
setappdata(f,'closed',0);
setappdata(f,'closedmod',-1);
setappdata(f,'mstore',zeros(200,5));
setappdata(f,'counter',0);
set(f,'KeyPressFcn',@KeyHandling);
setappdata(f,'playbackspeed',1);

%% Setting GUI elements
hclosed    = uicontrol('Style','pushbutton',...
             'String','Closed','Position',[100,120,100,100],...
             'Callback',{@closedbutton_Callback});
hopen    = uicontrol('Style','pushbutton',...
             'String','Open','Position',[100,20,100,100],...
             'Callback',{@openbutton_Callback});
hstart   = uicontrol('Style','pushbutton',...
             'String','Start','Position',[120,230,40,30],...
             'Callback',{@startbutton_Callback});
hcontour = uicontrol('Style','text',...
             'String','Duration','Position',[315,200,100,20]);
hextract = uicontrol('Style','pushbutton',...
            'String','Extract','Position',[315,20,100,20],...
             'Callback',{@extractbutton_Callback});
hstatus = uicontrol('Style','text',...
             'String','Where is the mouse?','Position',[315,150,100,20]);
hrestart = uicontrol('Style','pushbutton',...
             'String','RESTART','Position',[20,230,60,30],...
             'Callback',{@restart});
hfinish = uicontrol('Style','pushbutton',...
             'String','Finish','Position',[180,230,40,30],...
             'Callback',{@hfinished});
hdescript1 = uicontrol('Style','text',...
             'String','Playback Speed?','Position',[315,100,100,20]);
```

```
hplaybackspeed = uicontrol('Style','edit',...
             'Tag','framerater','Callback',{@plbs},...
             'Position',[345,80,40,20]);

set([f,hclosed,hopen,hcontour,hextract,hstart,hrestart,hfinish,hplaybackspeed
,hdescript1],'Units','normalized');


set(f,'Name','EPM Counter')

movegui(f,'center')

set(f,'Visible','on');

function KeyHandling(src,evnt)% assigns functions to key strokes
    switch evnt.Character
        case 'o'
            openbutton_Callback;
        case 'c'
            closedbutton_Callback;
        case 's'
            startbutton_Callback;
        case 'f'
            hfinished
    end
end

%% Setting functions assigned to buttons
function restart(src,evntdt)% restart button: restarts program memory
    setappdata(f,'closed',0);
    setappdata(f,'closedmod',-1);
    setappdata(f,'mstore',zeros(200,5));
    setappdata(f,'counter',0);
    set(hstatus,'String','Duration');
    set(hcontour,'String','Where is the mice?');
end

  function  startbutton_Callback(src,evntdt)%  start  button:  begins  time
counting
  mod=getappdata(f,'closedmod');
      if mod==-1
      tic %master tic
      c=getappdata(f,'counter');
```

```
      setappdata(f,'counter',c+1);
      set(hstatus,'String','on the center');
      end
      setappdata(f,'closedmod',0);
  end

  function hfinished(src,evntdt)% finish button: ends time counting and ends
last event
      b=toc;
      c=getappdata(f,'counter');
      store=getappdata(f,'mstore');
      store(c,5)=b;
      s=store(c,5)-store(c,4);
      n=get(hstatus,'String');
      n=sum(uint8(n));
      if n==926
          store(c,1)=s;
      end
      if n==1016
          store(c,2)=s;
      end
      if n==1247
          store(c,3)=s;
      end
        set(hstatus,'String','End');
        set(hcontour,'String','End');
      setappdata(f,'mstore',store);
  end

  function closedbutton_Callback(source,eventdata)% close button: first time
begins closed arm event and starts counting time, second time stops closed
arm event and stops counting time
   mod=getappdata(f,'closedmod');
  if mod==0
      c=getappdata(f,'counter');
      d=toc;
      set(hstatus,'String','on a closed');
      setappdata(f,'closedmod',1);
      store=getappdata(f,'mstore');
      c=getappdata(f,'counter');
      store(c,5)=d;
      store(c,3)=store(c,5)-store(c,4);
      s=store(c,3);
```

```
      c=c+1;
      store(c,4)=d;
      setappdata(f,'mstore',store);
      setappdata(f,'counter',c);
      setappdata(f,'closedmod',1);
      set(hcontour,'String',s);
  else
      d=toc;
      set(hstatus,'String','on the center');
      setappdata(f,'closedmod',0);
      store=getappdata(f,'mstore');
      c=getappdata(f,'counter');
      store(c,5)=d;
      store(c,2)=store(c,5)-store(c,4);
      s=store(c,2);
      c=c+1;
      store(c,4)=d;
      setappdata(f,'mstore',store);
      setappdata(f,'counter',c);
      set(hcontour,'String',s);
  end
  end


  function openbutton_Callback(source,eventdata)% open button: first time
begins open arm event and starts counting time, second time stops open arm
event and stops counting time
  mod=getappdata(f,'closedmod');
  if mod==0
      c=getappdata(f,'counter');
      d=toc;
      set(hstatus,'String','on an open');
      setappdata(f,'closedmod',1);
      store=getappdata(f,'mstore');
      c=getappdata(f,'counter');
      store(c,5)=d;
      store(c,3)=store(c,5)-store(c,4);
      s=store(c,3);
      c=c+1;
      store(c,4)=d;
      setappdata(f,'mstore',store);
      setappdata(f,'counter',c);
      setappdata(f,'closedmod',1);
```

```
        set(hcontour,'String',s);
    else
        d=toc;
        set(hstatus,'String','on the center');
        setappdata(f,'closedmod',0);
        store=getappdata(f,'mstore');
        c=getappdata(f,'counter');
        store(c,5)=d;
        store(c,1)=store(c,5)-store(c,4);
        s=store(c,1);
        c=c+1;
        store(c,4)=d;
        setappdata(f,'mstore',store);
        setappdata(f,'counter',c);
        set(hcontour,'String',s);
    end
    end

function extractbutton_Callback(source,eventdata)% extract button: outputs raw.csv with position and timestamps and destilled with desired values for further statistical analysis
    d=getappdata(f,'mstore');
    s=getappdata(f,'playbackspeed');
    d=d*s;
    folder=uigetdir
    cd(folder)

    dd=zeros(length(d),8);
    for i=1:length(d)
        if d(i,1)>0
            dd(1,1)=dd(1,1)+1;
        end
        if d(i,2)>0
            dd(1,2)=dd(1,2)+1;
        end
        if d(i,3)>0
            dd(1,3)=dd(1,3)+1;
        end
    end
    dd(2,1)=sum(d(:,1));
    dd(2,2)=sum(d(:,2));
    dd(2,3)=sum(d(:,3));
    dd(1,4)=dd(1,1)/(dd(1,1)+dd(1,2));
```

```
  dd(2,4)=dd(2,1)/(dd(2,1)+dd(2,2));

  csvwrite('raw.csv',d)
  csvwrite('destilled.csv',dd)
  set(hstatus,'String','Extracted!');
  set(hcontour,'String','Extracted!');
end

function plbs(obj,~)%defines playback speed of the video, which is used as a
multiplying factor for the timepoints registered by the program
        a=str2num(get(obj,'String'));
        setappdata(f,'playbackspeed',a);
end

end
```

3.8.2 Sourcecode for DLB counter application

```
function ldbcounter
%% Setting the common application variables
f = figure('Visible','off','Position',[360,500,450,285]);
setappdata(f,'closed',0);
setappdata(f,'closedmod',0);
setappdata(f,'mstore',zeros(50,5));
setappdata(f,'counter',1);
setappdata(f,'mclock',0);
setappdata(f,'difclock',0);
setappdata(f,'playbackspeed',1);
set(f,'KeyPressFcn',@KeyHandling);

%% Setting GUI elements
hdark    = uicontrol('Style','pushbutton',...
             'String','Dark','Position',[50,30,200,200],...
             'Callback',{@darkbutton_Callback});
hcontour = uicontrol('Style','text',...
             'String','Duration','Position',[315,200,100,20]);%   tells   the
duration of the last period on light or dark part
hextract = uicontrol('Style','pushbutton',...
            'String','Extract','Position',[315,20,100,20],...
             'Callback',{@extractbutton_Callback});
hstatus = uicontrol('Style','text',...
             'String','Where  is  the  mouse?','Position',[315,150,100,20]);%
tells the place of the animal at the moment
```

```
hrestart = uicontrol('Style','pushbutton',...
             'String','RESTART','Position',[20,230,60,30],...
             'Callback',{@restart});
hfinish = uicontrol('Style','pushbutton',...
             'String','Finish','Position',[180,230,40,30],...
             'Callback',{@hfinished});
hdescript1 = uicontrol('Style','text',...
             'String','Playback Speed?','Position',[315,100,100,20]);
hplaybackspeed = uicontrol('Style','edit','Callback',{@plbs},...
             'Tag','framerater',...
             'Position',[345,80,40,20]);

set([f,hdark,hcontour,hextract,hrestart,hfinish,hdescript1,hplaybackspeed],'U
nits','normalized');

set(f,'Name','Counter')

movegui(f,'center')

set(f,'Visible','on');

function KeyHandling(src,evnt)% assigns functions to key strokes
    switch evnt.Character
        case 'c'
            darkbutton_Callback;
        case 'f'
            hfinished
        case 'r'
            restart
    end
end

%% Setting functions assigned to buttons
function restart(src,evntdt)% restart button: restarts program memory
    setappdata(f,'closed',0);
    setappdata(f,'closedmod',0);
    setappdata(f,'mstore',zeros(50,5));
    setappdata(f,'counter',1);
    setappdata(f,'mclock',0);
    setappdata(f,'difclock',0);
    set(hstatus,'String','Duration');
    set(hcontour,'String','Where is the mice?');
end
```

```
  function darkbutton_Callback(source,eventdata) %transition button: starts time counting at first stroke and then defines where the animal is and starts and ends counting the time it spends on the a particular part of the maze
  mod=getappdata(f,'closedmod');
  if mod==0
      tic;
      set(hstatus,'String','on the dark side');
      setappdata(f,'closedmod',2);
      set(hdark,'String','Into the Light');
  else
      if mod==1;
          b=toc
          set(hstatus,'String','on the dark side');
          store=getappdata(f,'mstore');
          c=getappdata(f,'counter');
          store(c,4)=b;
          store(c,1)=store(c,4)-store(c,3);
          set(hcontour,'String',store(c,1));
          store(c+1,3)=b;
          setappdata(f,'mstore',store);
          setappdata(f,'counter',c+1);
          setappdata(f,'closedmod',2);
          set(hdark,'String','Into the Light')
      else
          b=toc
          set(hstatus,'String','on the light side');
          store=getappdata(f,'mstore');
          c=getappdata(f,'counter');
          store(c,4)=b;
          store(c,2)=store(c,4)-store(c,3);
          set(hcontour,'String',store(c,2));
          store(c+1,3)=b;
          setappdata(f,'mstore',store);
          setappdata(f,'counter',c+1);
          setappdata(f,'closedmod',1);
          set(hdark,'String','Into the Dark');
      end
  end
  end

  function hfinished(src,evntdt)%finish button: terminates the time counting
      b=toc
```

```
    c=getappdata(f,'counter');
    store=getappdata(f,'mstore');
    store(c,4)=b;
    if store(c-1,1)>0
        store(c,2)=store(c,4)-store(c,3);
    else
        store(c,1)=store(c,4)-store(c,3);
    end
    set(hstatus,'String','End');
    set(hcontour,'String','End');
    setappdata(f,'mstore',store);
end

function extractbutton_Callback(source,eventdata)%extract button: outputs raw.csv with position and timestamps and destilled with desired values for further statistical analysis
d=getappdata(f,'mstore');
s=getappdata(f,'playbackspeed');
d=d*s;
folder=uigetdir
cd(folder)

dd=zeros(length(d),8);
for i=1:length(d)
    if d(i,1)>0
        dd(1,1)=dd(1,1)+1;
    end
    if d(i,2)>0
        dd(1,2)=dd(1,2)+1;
    end
end
dd(1,3)=sum(d(:,1));
dd(1,4)=sum(d(:,2));
dd(1,6)=d(1,4);


csvwrite('raw.csv',d)
csvwrite('destilled.csv',dd)
set(hstatus,'String','Extracted!');
set(hcontour,'String','Extracted!');
end

function plbs(obj,~)%defines playback speed of the video, which is used as
```

```
a multiplying factor for the timepoints registered by the program
        a=str2num(get(obj,'String'));
        setappdata(f,'playbackspeed',a);
  end

end
```

3.8.3 Sourcecode for Open-field Heatmap creator

The OF files handling and central algorithm for the generation of the heatmap matrix were done with the kind help of Bruno Cruz.

```
%% Selection of the folder containing the OF data for one experimental
condition
folder=uigetdir;
Resolution=27+1; %number of divisions of the grid
GGV=zeros(Resolution);
cd(folder) %roots the path
files = dir('*.txt');
nfiles=0;
Tcrossings=0;
%% Analysis of each file
for file = files'
    %% conversion of the OF data file into analyzable matrices
    txt=(file.name);
    VarName1=ofreader(txt);
    a= strrep(VarName1, ',', '.');
    XX=zeros(size(a,1),1);
    YY=zeros(size(a,1),1);
    countx=1;
    county=1;
    for ii=1:9000
        cvx=a(ii,3);
        cvx=char(cvx);
        cvx=str2num(cvx);
        XX(ii)=cvx;
    end
    for jj=1:9000
        cvy=a(jj,4);
        cvy=char(cvy);
        cvy=str2num(cvy);
        YY(jj)=cvy;
    end
```

```
    %% conversion of XY positions over time into frequencies on a two
dimensional grid
    GridValues=zeros(Resolution);
    i=1;
    cy=Resolution+2;
    cx=Resolution+2;
    Checkmatrixy=linspace(0,max(YY),Resolution);
    Checkmatrixx=linspace(0,max(XX),Resolution);
    while i<=9000
        x=XX(i);
        y=YY(i);
        while isnan(x) || isnan(y)
            i=i+1;
            x=XX(i);
            y=YY(i);
        end
        qx=1;
        qy=1;
        while x>Checkmatrixx(qx+1)
            qx=qx+1;
        end
        while y>Checkmatrixy(qy+1)
            qy=qy+1;
        end
        GridValues(qx,qy)=GridValues(qx,qy)+1;
        i=i+1;
        cy=qy;
        cx=qx;
    end
    GGV=GGV+GridValues;
    nfiles=nfiles+1;
end
```

ofreader function used on the previous script

```
function S=ofreader(file)
%selection of relevant data on OF files for heatmap generation
f=fopen(file, 'r');
S=textscan(f,'%s %s %s %s %s', 'headerlines', 37);
S=[S{1,:}];
S=S(1:end-1,1:5);
fclose(f)
```

Individual script to create the heatmaps

```
%% Creates a single heatmap by subtracting the control to the test condition
ofhtmapper
ac=GGV;
ofhtmapper
bc=GGV;
bc=log(bc);
ac=log(ac);
c=ac-bc;
c(~isfinite(c))=0;
[cx cy]=meshgrid(1:0.028:28,1:0.028:28);
ci=interp2(c,cx,cy);
HeatMap(ci,'Colormap',jet);
colormapeditor
```

3.8.4 Sourcecode for Grooming and Rearing counter application

```
function GRcounter

f = figure('Visible','off','Position',[360,500,450,285]);
setappdata(f,'closed',0);
setappdata(f,'closedmod',0);
setappdata(f,'mstore',zeros(70,5));
setappdata(f,'counter',1);
setappdata(f,'mclock',0);
setappdata(f,'difclock',0);
setappdata(f,'playbackspeed',1);
set(f,'KeyPressFcn',@KeyHandling);

hgrooming    = uicontrol('Style','pushbutton',...
              'String','Grooming','Position',[50,100,100,100],...
              'Callback',{@groomingbutton_Callback});
hrearing    = uicontrol('Style','pushbutton',...
              'String','Rearing','Position',[150,100,100,100],...
              'Callback',{@rearingbutton_Callback});
hcontour = uicontrol('Style','text',...
              'String','Duration','Position',[315,200,100,20],...
              'Callback',{@contourtext_Callback});
hextract = uicontrol('Style','pushbutton',...
             'String','Extract','Position',[315,20,100,20],...
              'Callback',{@extractbutton_Callback});
```

```
hstatus = uicontrol('Style','text',...
             'String','Where is the mouse?','Position',[315,150,100,20],...
             'Callback',{@statustext_Callback});
hrestart = uicontrol('Style','pushbutton',...
             'String','RESTART','Position',[20,230,60,30],...
             'Callback',{@restart});
hstart   = uicontrol('Style','pushbutton',...
             'String','Start','Position',[120,230,40,30],...
             'Callback',{@startbutton_Callback});
hfinish = uicontrol('Style','pushbutton',...
             'String','Finish','Position',[180,230,40,30],...
             'Callback',{@hfinished});
hdescript1 = uicontrol('Style','text',...
             'String','Playback Speed?','Position',[315,100,100,20]);
hplaybackspeed = uicontrol('Style','edit','Callback',{@plbs},...
             'Tag','framerater',...
             'Position',[345,80,40,20]);

set([f,hcontour,hextract,hrestart,hfinish,hdescript1,hplaybackspeed,hgrooming
,hrearing,hstart],'Units','normalized');



set(f,'Name','Counter')

movegui(f,'center')

set(f,'Visible','on');

function KeyHandling(src,evnt)
    switch evnt.Character
        case 's'
            startbutton_Callback;
        case 'g'
            groomingbutton_Callback;
        case 'r'
            rearingbutton_Callback;
        case 'f'
            hfinished;
        case 'r'
            restart;
    end
end
```

```
function startbutton_Callback(src,evntdt)
  mod=getappdata(f,'closedmod');
      if mod==0
      tic
      set(hcontour,'String','Started');
      end
      setappdata(f,'closedmod',0);
end

function restart(src,evntdt)
    setappdata(f,'closed',0);
    setappdata(f,'closedmod',0);
    setappdata(f,'mstore',zeros(70,5));
    setappdata(f,'counter',1);
    setappdata(f,'mclock',0);
    setappdata(f,'difclock',0);
    set(hstatus,'String','Duration');
    set(hcontour,'String','Where is the mice?');
end

  function hfinished(src,evntdt)
      b=toc
      c=getappdata(f,'counter');
      store=getappdata(f,'mstore');
      store(c,3)=b;
      set(hstatus,'String','End');
      set(hcontour,'String','End');
      setappdata(f,'mstore',store);
  end

  function groomingbutton_Callback(source,eventdata)
  mod=getappdata(f,'closedmod'); %grooming button: one ‘g’ keystroke to start
the event and a second ‘g’ keystroke to end the event;
      if mod==0;
          b=toc
          set(hstatus,'String','grooming...');
          store=getappdata(f,'mstore');
          c=getappdata(f,'counter');
          store(c,3)=b;
          setappdata(f,'mstore',store);
          setappdata(f,'closedmod',1);
      else
          b=toc
```

```
          set(hstatus,'String','just groomed');
          store=getappdata(f,'mstore');
          c=getappdata(f,'counter');
          store(c,4)=b;
          store(c,1)=store(c,4)-store(c,3);
          set(hcontour,'String',store(c,1));
          setappdata(f,'mstore',store);
          setappdata(f,'counter',c+1);
          setappdata(f,'closedmod',0);
      end
  end


function rearingbutton_Callback(source,eventdata) % rearing button: one 'r'
keystroke to initiate event and a second 'r' keystroke to end the event;
  mod=getappdata(f,'closedmod');
      if mod==0;
          b=toc
          set(hstatus,'String','rearing...');
          store=getappdata(f,'mstore');
          c=getappdata(f,'counter');
          store(c,3)=b;
          setappdata(f,'mstore',store);
          setappdata(f,'closedmod',1);
      else
          b=toc
          set(hstatus,'String','just reared');
          store=getappdata(f,'mstore');
          c=getappdata(f,'counter');
          store(c,4)=b;
          store(c,2)=store(c,4)-store(c,3);
          set(hcontour,'String',store(c,2));
          store(c+1,3)=b;
          setappdata(f,'mstore',store);
          setappdata(f,'counter',c+1);
          setappdata(f,'closedmod',0);
      end
  end

function contourtext_Callback(source,eventdata)
end

function statustext_Callback(scr,evndt)
```

```
end

  function extractbutton_Callback(source,eventdata) %extract button: outputs
raw.csv with event timestamps and destilled with desired values for further
statistical analysis
  d=getappdata(f,'mstore');
  s=getappdata(f,'playbackspeed');
  d=d*s;
  folder=uigetdir
  cd(folder)

  dd=zeros(length(d),8);
  for i=1:length(d)
      if d(i,1)>0
          dd(1,1)=dd(1,1)+1;
      end
      if d(i,2)>0
          dd(1,2)=dd(1,2)+1;
      end
  end
  dd(1,3)=sum(d(:,1));
  dd(1,4)=sum(d(:,2));


  csvwrite('raw.csv',d)
  csvwrite('destilled.csv',dd)
  set(hstatus,'String','Extracted!');
  set(hcontour,'String','Extracted!');
  end

  function plbs(obj,~)
        a=str2num(get(obj,'String'));
        setappdata(f,'playbackspeed',a);
  end
end
```

Chapter 3 – Results

4 RESULTS

4.1 AN ABRUPT DECREASE OF GHRELIN LEVELS AFFECTS THE MOLECULAR COMPOSITION OF THE POST-SYNAPTIC DENSITY OF HIPPOCAMPAL NEURONS

4.1.1 GluA1 levels on the post-synaptic density are affected by the abrupt decrease of ghrelin levels

Previous work by Luís Ribeiro unveiled the effects of increasing levels of ghrelin on AMPA receptor subunit composition change in hippocampal synapses {Ribeiro, 2014 #1485}. To date, however, the direct effects of an abrupt decrease in ghrelin levels, as seen in the peri/postprandial period remains unexplored. To simulate and study a physiologically relevant decrease in ghrelin signaling, the subsequent protocol was implemented: ghrelin was applied for 5 hours being renewed at hour 2 and 4 during the protocol; at 4h30 ghrelin was either removed, by substituting the culture media with ghrelin-free culture media, or maintained, with the culture media being manipulated. For each of these two conditions there was a respective control condition with no ghrelin being added (4 conditions in total). At the end of the protocol we proceeded with post-synaptic density purification. The timing of the protocol is illustrated in figure 13.

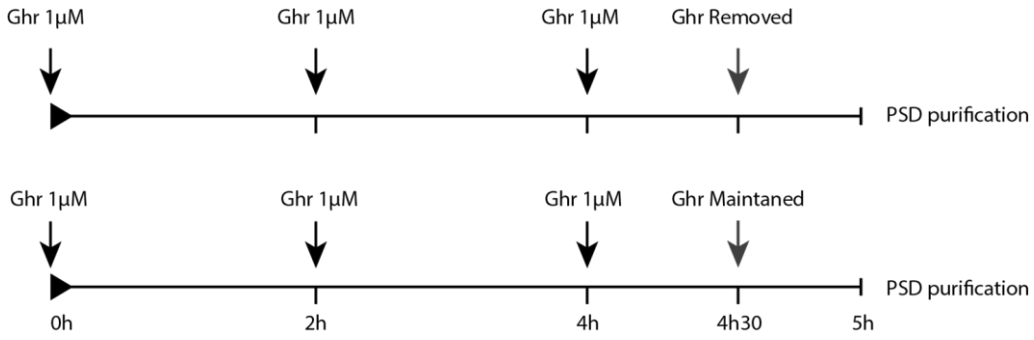


Figure 11 - Abrupt Ghrelin Removal protocol diagram

We then analyzed the purified fractions through western blot. From now on the removed conditions are represented by light blue color and the non-removed conditions are represented by dark-blue color. We observed a consistent decrease in the levels of GluA1 in the post-synaptic density fractions of cultures where ghrelin was abruptly removed, figure 14. Under the same conditions, cultures where ghrelin was not abruptly removed, showed a slight tendency towards increased levels of GluA1 in the post-synaptic density fractions, however this was not statistically significant.

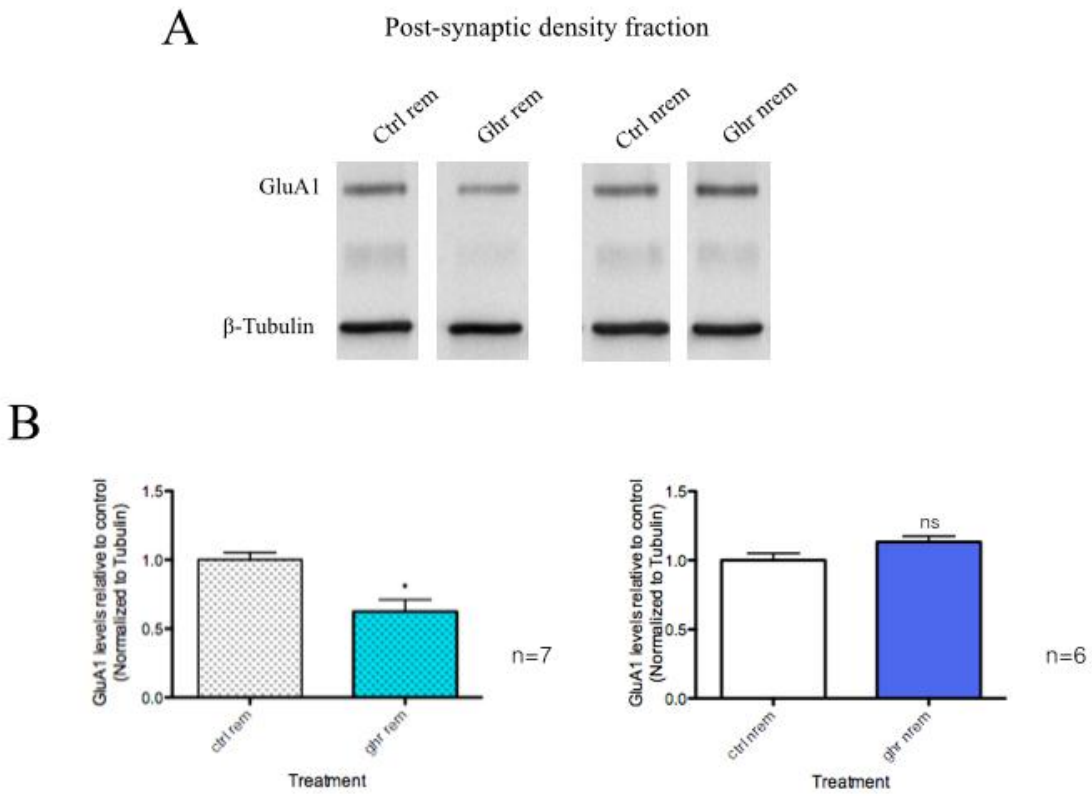


Figure 12 – Effect of abrupt removal of ghrelin on the levels of GluA1 in post-synaptic densities. A: Representative blots for GluA1 and tubulin (loading control), for the conditions where ghrelin was abruptly removed (Ghr rem-Ghrelin removed; Ctrl rem-Control for removal) and where ghrelin was not removed (Ghr nrem-Ghrelin not removed;Ctrl nrem-Control for no removal). **B**: Mean intensity of GluA1 (normalized to tubulin). There is a significant decrease in GluA1 levels for the ghrelin removal condition in comparision to control, as assessed by Paired t-test (* $P < 0.05$). There is no significant change in the levels of GluA1 for the condition where ghrelin was not removed. Error bars represent s.e.m and n represents the number of independent experiments.

We then analyzed the levels of GluA1 on total hippocampal lysate fractions. These levels reflect the entire cellular expression of GluA1. In the conditions where ghrelin was removed, we did not observe significant differences on the levels of GluA1 in comparison to control (figure 15). In the conditions where ghrelin was not removed, there is a strong tendency towards an increase in GluA1 levels. Taking all these results into consideration, we can conclude that ghrelin induces an increase in the expression of GluA1 after slices are exposed to ghrelin for 5h, but this is not directly reflected on an increase in levels of GluA1 on post-synaptic densities. However, if ghrelin is abruptly removed 30 minutes before these 5h, there is a significant decrease of GluA1 levels at post-synaptic densities. We hypothesize that these

GluA1 receptors are degraded during this 30-minute period, to such a degree that the overall cellular levels of GluA1 become indistinguishable from those of non-ghrelin stimulated neurons.

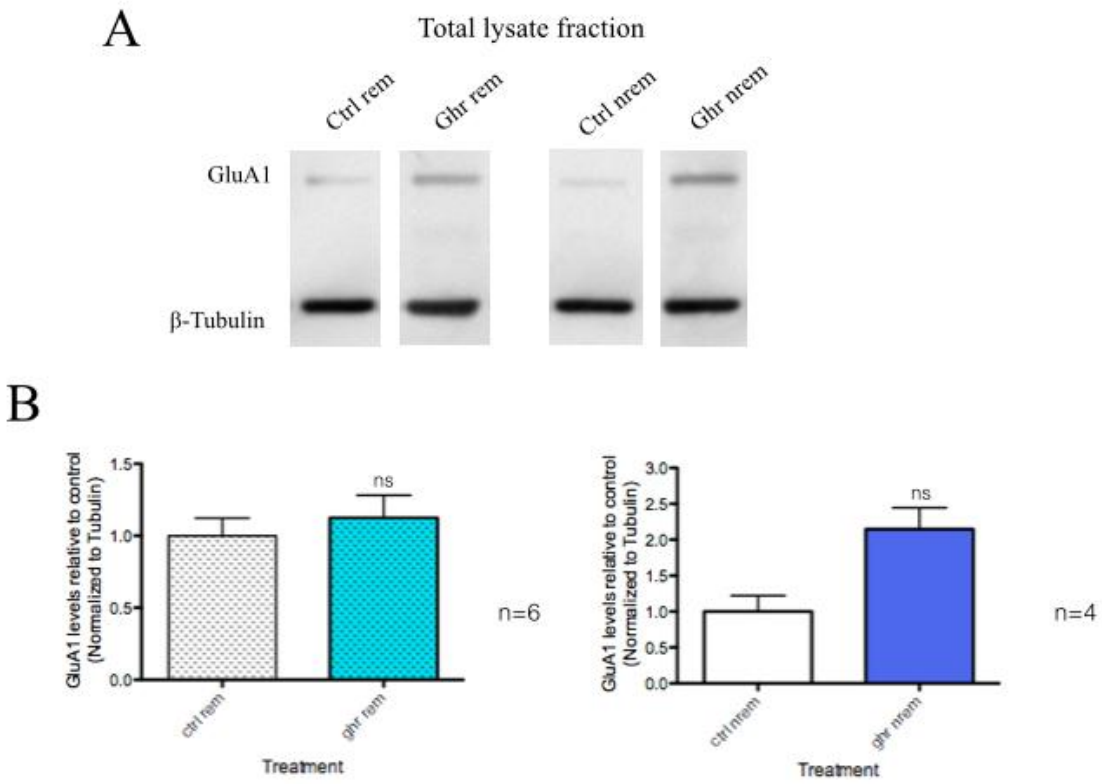


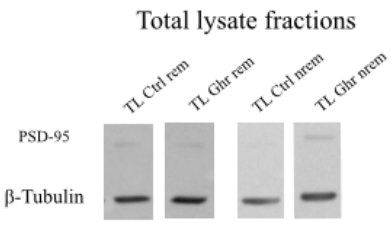
Figure 13 - Effect of abrupt removal of ghrelin on GluA1 levels on the total lysate fractions. A: Representative blots for GluA1 and tubulin (loading control), for the conditions where ghrelin was abruptly removed (Ghr rem-Ghrelin removed; Ctrl rem-Control for removal) and where ghrelin was not removed (Ghr nrem-Ghrelin not removed; Ctrl nrem-Control for no removal). **B**: Mean intensity of GluA1 (normalized to tubulin). There are no significant changes as assessed by paired t test. There is a strong tendency towards an increase in GluA1 levels for the ghrelin non removal condition in comparision to control. Error bars represent s.e.m and n represents the number of independent experiments.

4.1.2 Whole cell PSD-95 expression levels show a tendency towards an increase after treatment with ghrelin

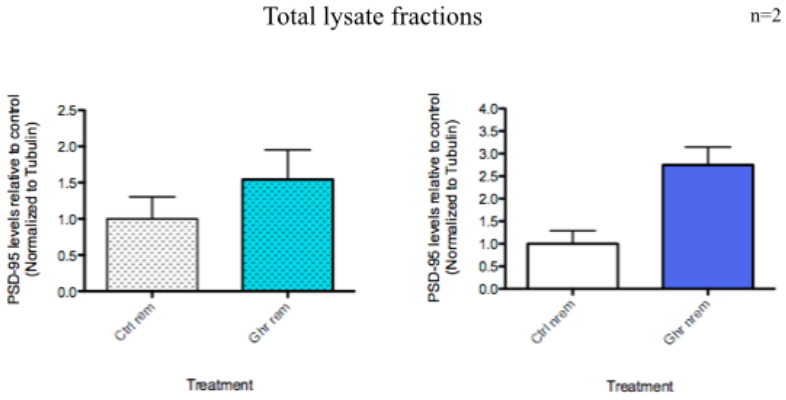
We next analyzed whether the levels of PSD-95 changed after abrupt removal of ghrelin, both at the level of total lysates and post-synaptic densities. Previous work done by Fiona van Leeuwen in the laboratory of Ana Luísa Carvalho showed that activation of the GHSR1a in mature hippocampal neurons increased the levels of PSD-95 clusters, as assessed by immunocytochemistry (unpublished data). We

also observed a tendency towards increased levels of PSD-95 in total lysate fractions, regardless of removal of ghrelin, and did not observe differences in the levels of PSD-95 in PSD fractions (figure 16). We may conclude that GHS-R1a activation for 4h30 increases the expression of PSD-95 and that the abrupt removal of ghrelin does not measurably affect the levels of PSD-95 in our time window. This finding corroborates the observations of Fiona van Leeuwen.

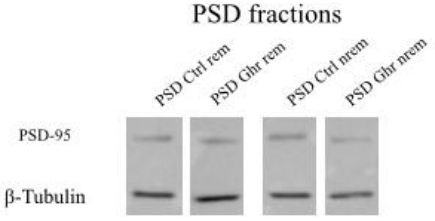
A


Total lysate fractions
TL Ctrl rem
TL Ghr rem
TL Ctrl nrem
TL Ghr nrem
PSD-95
β-Tubulin

B


Total lysate fractions
n=2
PSD-95 levels relative to control (Normalized to Tubulin)
Ctrl rem
Ghr rem
Treatment
PSD-95 levels relative to control (Normalized to Tubulin)
Ctrl nrem
Ghr nrem
Treatment

C


PSD fractions
PSD Ctrl rem
PSD Ghr rem
PSD Ctrl nrem
PSD Ghr nrem
PSD-95
β-Tubulin

D

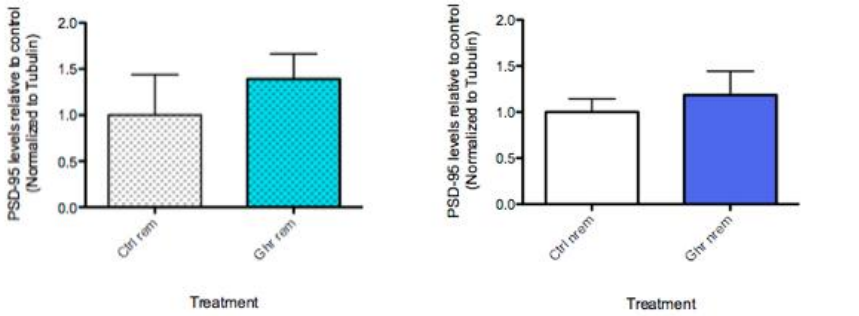
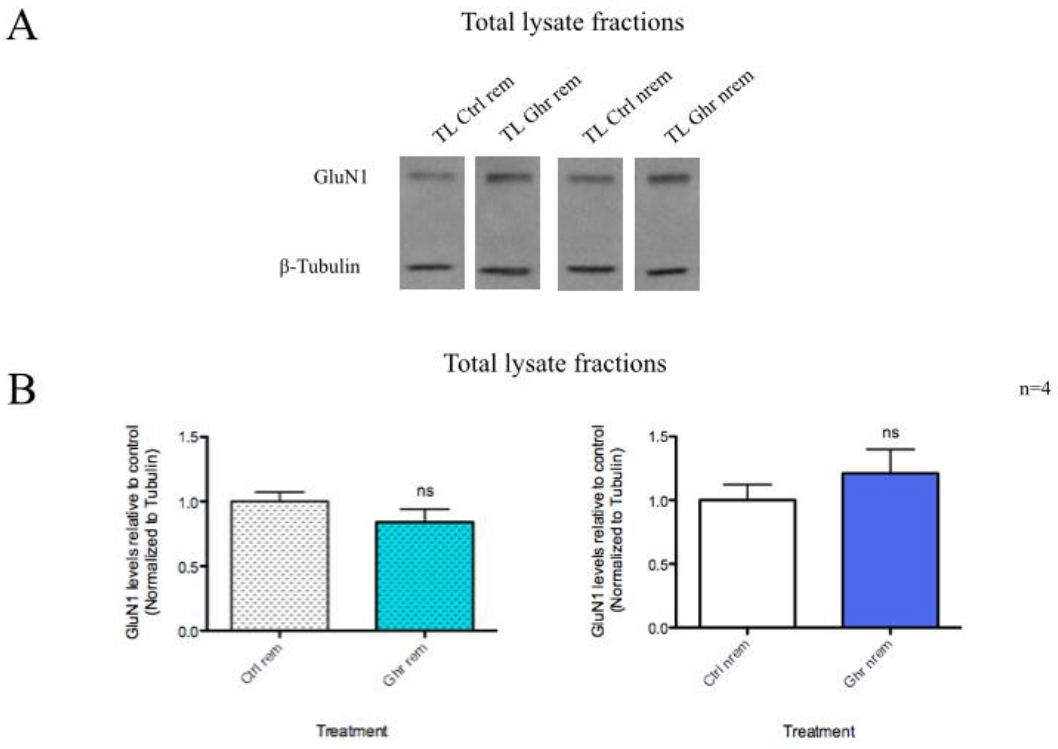

PSD fractions
n=2
PSD-95 levels relative to control (Normalized to Tubulin)
Ctrl rem
Ghr rem
Treatment
PSD-95 levels relative to control (Normalized to Tubulin)
Ctrl nrem
Ghr nrem
Treatment

Figure 16 - Effect of abrupt decrease of ghrelin on whole cell and post-synaptic density levels of PSD-95 A,C: Representative blots for PSD-95 and tubulin (loading control), of total lysate (A) and PSD fractions (C) for the conditions where ghrelin was abruptly removed (Ghr rem-Ghrelin removed; Ctrl rem-Control for removal) and where ghrelin was not removed (Ghr nrem-Ghrelin not removed;Ctrl nrem-Control for no removal). **B,D**: Mean intensity of PSD-95 (normalized to tubulin). There is a strong tendency towards an increase in PSD-95 levels on total lysate fractions (B) in the presence of ghrelin, regardless of removal. There are no significant changes in the PSD fractions as assessed by paired t tests. Error bars represent s.e.m and n represents the number of independent experiments.

4.1.3 NMDA receptor GluN1 expression levels are not affect by abrupt removal or the treatment with ghrelin

To further analyze how the post-synaptic density composition was affected by the abrupt decrease of ghrelin, we analyzed the levels of NMDA receptor subunit GluN1 on total lysate and post-synaptic density fractions of previously obtained samples. We observed that neither 5h of treatment with ghrelin nor the abrupt removal of ghrelin significantly altered the levels GluN1 in the total lysates or PSD fractions (figure 17).



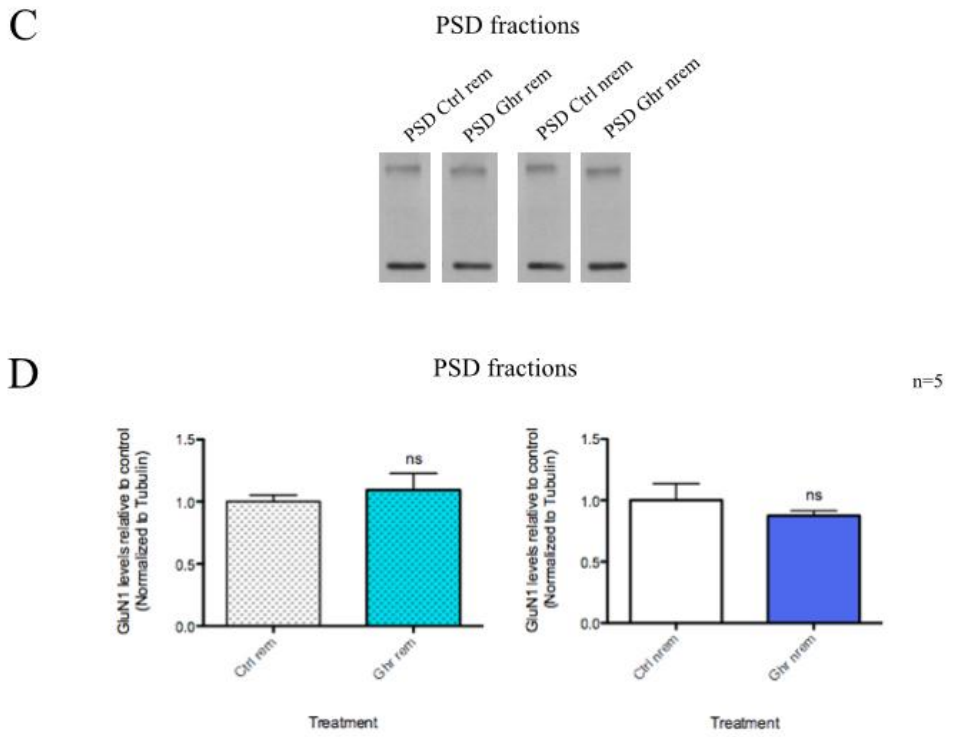


Figure 14 - Effect of abrupt decrease of ghrelin on whole cell and post-synaptic density levels of GluN1. Representative blots for GluN1 and tubulin (loading control), of total lysate (**A**) and PSD fractions (**C)** for the conditions where ghrelin was abruptly removed (Ghr rem-Ghrelin removed; Ctrl rem-Control for removal) and where ghrelin was not removed (Ghr nrem-Ghrelin not removed; Ctrl nrem-Control for no removal). Mean intensity of GluN1 (normalized to tubulin) for total lysate (**B**) and PSD fractions (**D)**. There are no significant differences between conditions treated and controls, as assessed by paired t tests. Error bars represent s.e.m and n represents the number of independent experiments.

4.2 GHRELIN DECREASES ANXIETY REGARDLESS OF THE DURATION OF THE INTERVAL BETWEEN DRUG ADMINISTRATION AND THE START OF THE TEST

4.2.1 Ghrelin decreases anxiety on the open-field test if animals are tested 45 min after its administration, and decreases motor activity if animals are tested 10 min after its administration

To test the hypothesis that ghrelin effects on anxiety are dependent on the duration of the interval between ghrelin administration and the start of the test, we began by performing the open-field test. After intraperitoneal administration of 2 μg/g of ghrelin, we waited 10 or 45 minutes before starting the behavioral test. The experimental conditions are represented in figure 18; we performed controls for each condition (a total of 4 conditions). From now on the 10 min condition is represent by red color and the 45 min condition is represented by orange color. Evaluating the anxiety levels through the time animals spends on the center area of the arena, we observed that for the 10 minutes condition, ghrelin induced a decrease in the time spent in the center area, and for the 45 minutes condition ghrelin increased the time spent on the center area (A,C figure 19). We also observed that the total distance travelled by the animals was decreased for the animals injected with ghrelin in the 10 minutes condition and unchanged in the 45 minutes condition (B,D figure 19). We propose this may explain the decrease in the time spent in the center area, since decreased motility by itself, may reduce the chances of animals venturing into the more exposed part of the open-field. If we average the sums of the distance and time values for the first 10, 30 and 45 minutes of each animal in each condition, the previously suggested differences become more evident (figure 19). The analysis of the position of the mice in the open-field overtime through heatmaps (figure 20) also suggests the same tendencies. From these results we propose that the previously described anxiogenic effect of ghrelin may partially be the result of the effect of a decrease in motor activity. At the 45 minutes time-point there is a trend towards longer time spent in the center area, suggesting that ghrelin is produces an anxiolytic effect.

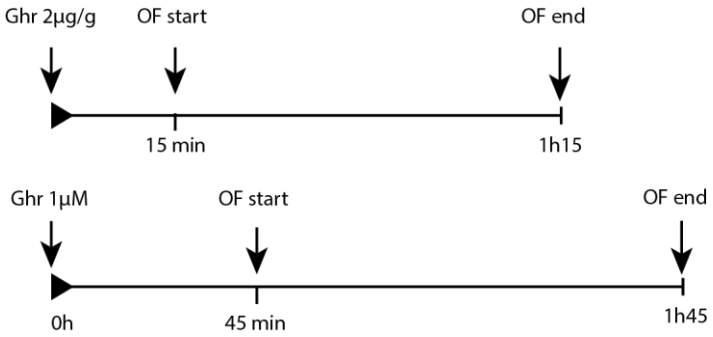


Figure 15 - Open-Field test timing

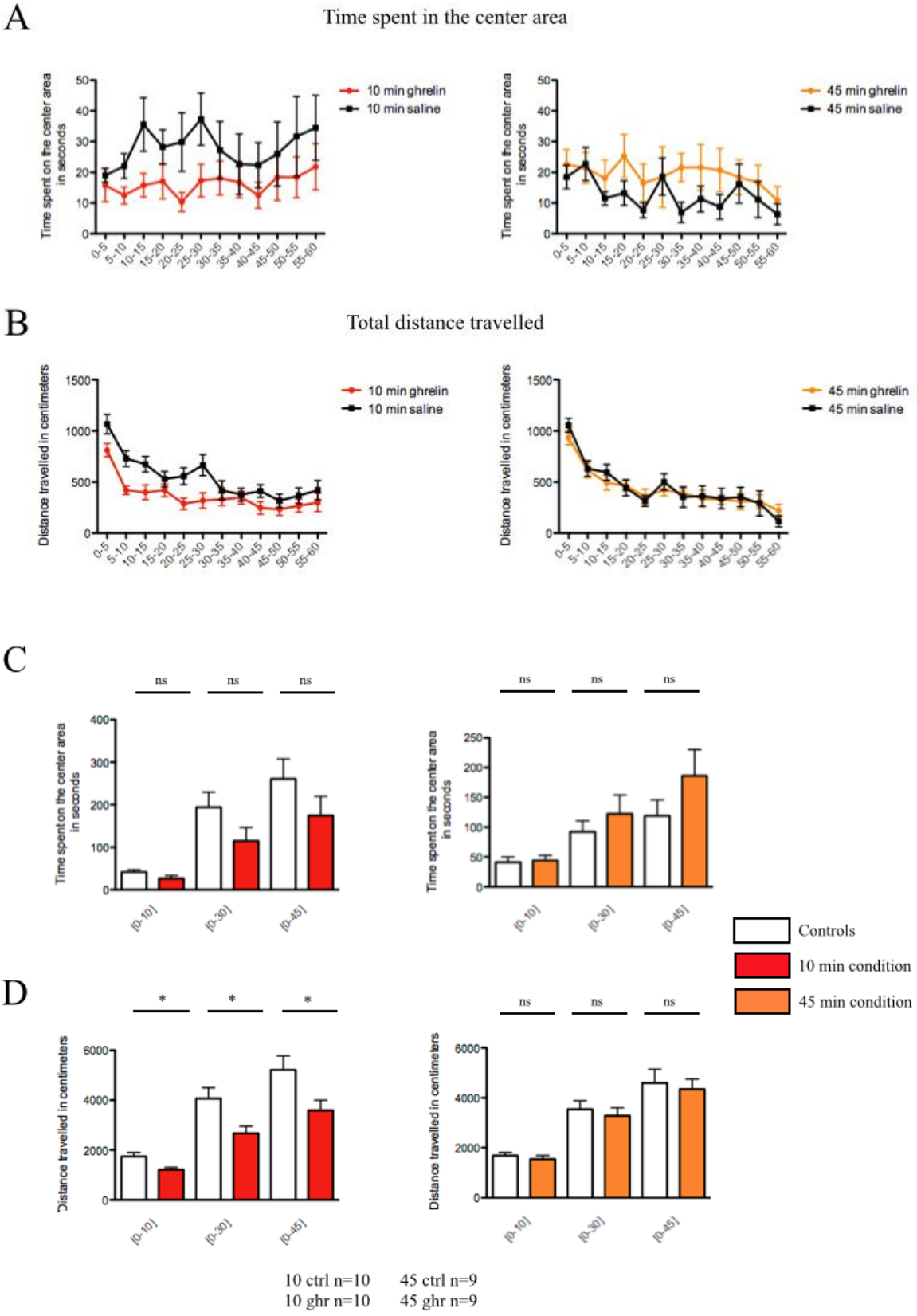

A
Time spent in the center area
Time spent on the center area in seconds
10 min ghrelin
10 min saline
45 min ghrelin
45 min saline
B
Total distance travelled
Distance travelled in centimeters
C
ns
D
*
[0-10]
[0-30]
[0-45]
Controls
10 min condition
45 min condition
10 ctrl n=10
10 ghr n=10
45 ctrl n=9
45 ghr n=9

Figure 19 – Effect of ghrelin on anxiety as evaluated by the open-field test. A, B: Time spent in the center area (A) and total distance travelled (B) in the open-field, plotted as average values during increments of 5 minutes for the duration of the test (60 minutes). For the 10 minutes condition, animals injected with ghrelin have a tendency to spend less time in the center area and show lower motor activity, and for the 45 minutes condition, animals injected with ghrelin have a tendency to spend more time in the center area with the motor activity being unchanged. Two-way ANOVA test with Bonferroni *post hoc* test showed statistical differences in the case of 5-10 and 20-25 increment on motor activity for the animals injected with ghrelin, for the 10 minutes condition (* $P < 0.05$) **C,D**: Time spent in the center area (C) and total distance travelled (D) in the open-field, presented as the average of the sums of the values for the first 10, 30 and 45 minutes, for each animal in each condition. These tendencies reinforce the increase in time spent in the center area for the 45 minutes condition, and decreased motor activity and time spent on the center area in the 10 minutes condition. Unpaired t-student test showed statistical differences between several groups (* $P < 0.05$). For the whole panel, error bars represent s.e.m and n represents the number of animals.

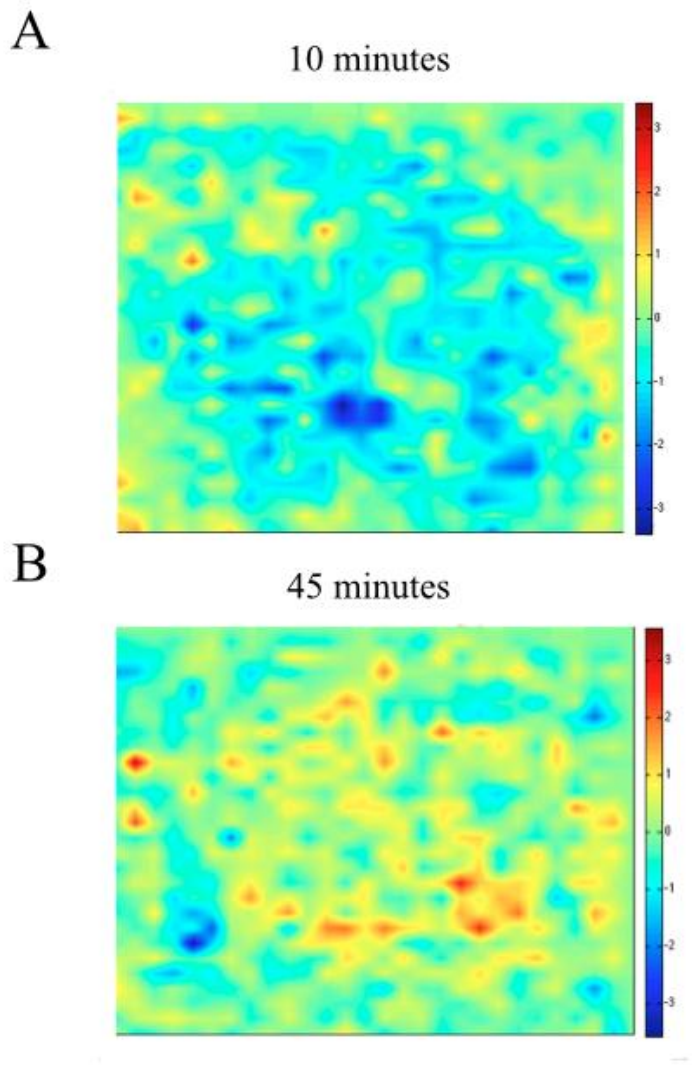
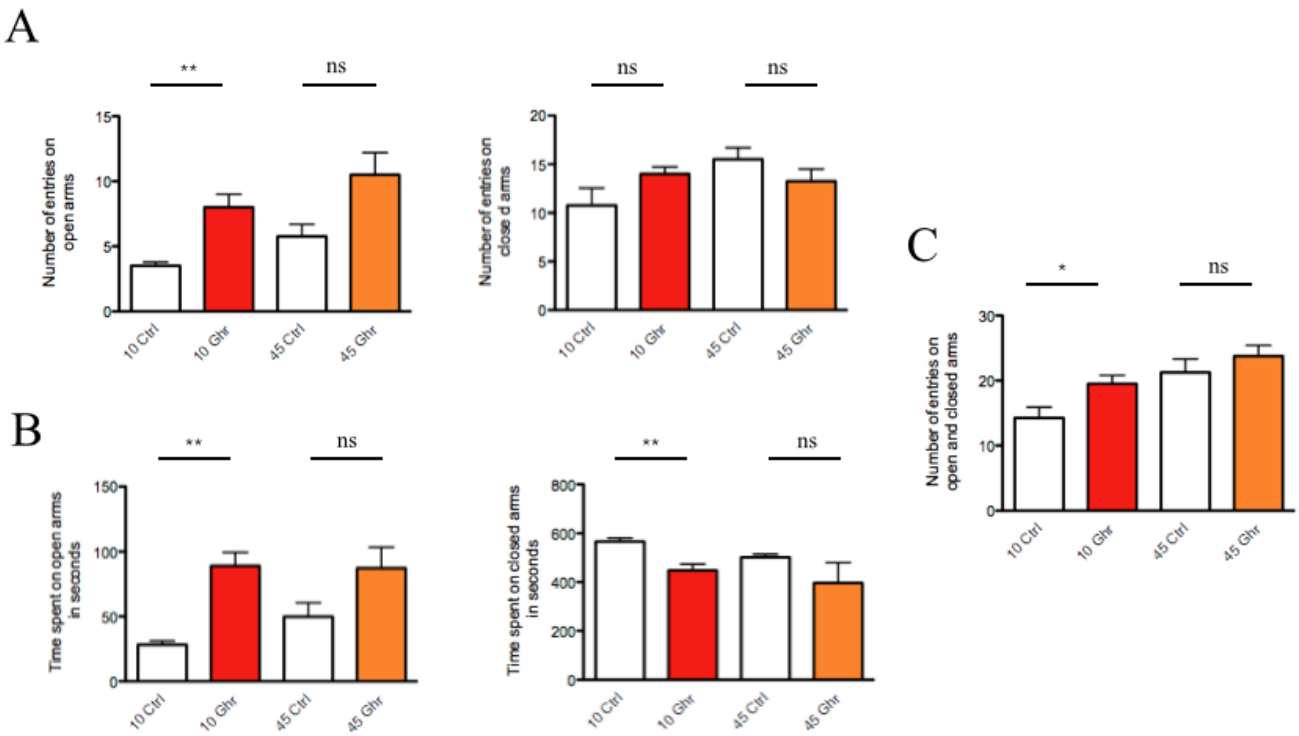


Figure 16 - Heatmaps of the open-field test for ghrelin and saline treated animals. The XY position of mice over time was converted into the frequency of the presence of the animals on a two-dimensional matrix representing the open-field arena. These matrices were summed for all the animals in each condition and the values were converted into the logarithmic scale. The matrix for the control conditions was subsequently substracted from the ghrelin conditions: values above 0 represent areas where animals treated with ghrelin were more present and values below 0 represent areas where animals treated with ghrelin were more absent, in comparison to control. **A** represents the 10 minute conditions and **B** represents the 45 minute conditions. These heatmaps indicate that animals treated with ghrelin, for the 10 minutes group, spent less time in the center and for the 45 minutes spent more time on the center, in comparison to control. n=10 for 10 min conditions and n=9 for 45 minutes condtions.

4.2.2 Ghrelin decreases anxiety on the elevated-plus maze test regardless of animals being tested 10 or 45 minutes after its administration

To further elucidate the effects of ghrelin on anxiety, we tested animals in an elevated-plus maze test. The wait times between giving i.p. injections of ghrelin and starting the tests was the same as for the open-field test. In this test, anxiety is measured by the proportion of activity in the open arms of the maze, namely the number of entries and time spent in these arms, and the percentage of these values regarding the number of entries and time spent in both types of arms (Matsuo et al., 2010; Stein and Steckler, 2010). In both 10 and 45 minutes conditions, ghrelin increased the time spent and number of entries in open-arms (figure 21). At the 45 minute condition there were no statistically significant differences. In the case of motor activity, which in this test can be evaluated through the number of entries in the closed arms and the total number of entries (Stein and Steckler, 2010), there was a significant increase in total number or entries for the 10 minutes group (C, figure 21), without differences in the number of entries in the closed arms for both groups (A, figure 21), suggesting a slight increase in motor activity. The percentages of time spent and number of entries on the open-arms further show that ghrelin decreases anxiety in the test (D, E, figure 21), regardless of the time at which the test is started, after the injection of ghrelin is given.



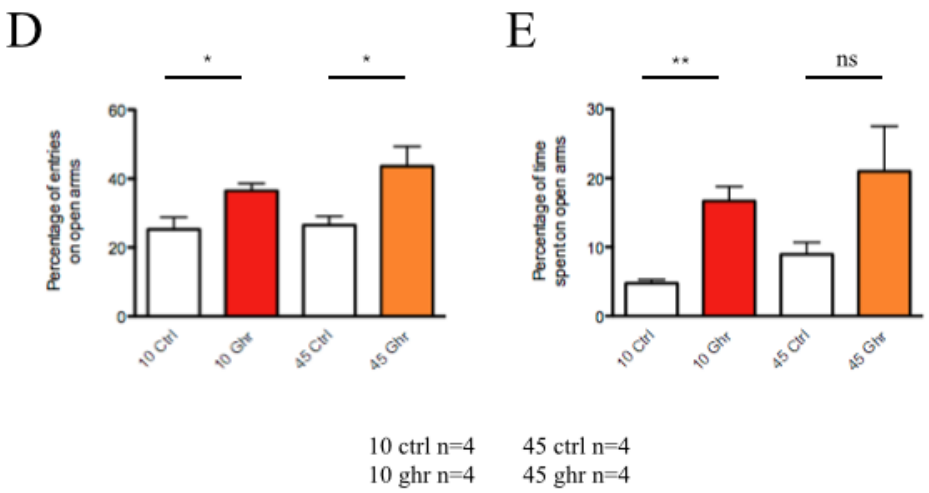


Figure 17 - Effect of ghrelin on anxiety as evaluated by the elevated-plus maze test. (10 Ctrl/Ghr: 10 min condition control and ghrelin treated; 45 Ctrl/Ghr: 45 min condition control and ghrelin treated) **A**: Number of entries in open and closed arms. Ghrelin significantly increases the number of entries on open arms for the 10 minutes condition and shows a tendency to also increase the number of open arm entries for the 45 minutes condition. There are no differences regarding closed arm entries. **B**: Time spent in open and closed arms. Ghrelin significantly increase the time spent in the open arms and decreases the time spent in closed arms, in the 10 minutes condition. For the 45 minutes condition, ghrelin shows a tendency similar to the 10 minutes condition. **C**: Sum of entries on closed and open arms. Ghrelin significantly increased the number of entries in both types of arms for the 10 minutes condition, without having an effect for the 45 minutes condition. **D**: Percentage of entries in the open arms expressed by the number of entries in the open arms divided by the sum of the number of entries in the open and closed arms and multiplied by 100. Ghrelin increases this percentage in both 10 and 45 minutes condition. **E**: Percentage of time spent in the open arms expressed by the time in the open arms divided by the sum of the time in the open and closed arms and multiplied by 100. Ghrelin significantly increases this percentage in the case of the 10 minutes condition and shows a tendency towards increase in the 45 minutes condition. Statistics was done with unpaired t-student test (* $P < 0.05$; ** $P < 0.01$). Error bars represent s.e.m and n represents the number of independent animals.

4.2.3 Ghrelin decreases anxiety on the dark-light box test regardless of animals being tested 10 or 45 minutes after its administration

To clarify whether the anxiogenic effects observed on the open-field test could be observed on another type of behavioral test, we performed the light-dark box test. This test is based on the rodents' natural aversion to illuminated spaces, and so anxiety can be evaluated by the number of transitions between the light and dark compartment and the time spent exploring the light compartment (Stein and Steckler, 2010). In agreement with the results obtained on the elevated-plus maze test, ghrelin decreases anxiety in both 10 and 45 minutes groups, although without statistical significance on the number of transitions (figure 22). This was most evident on the time spent in each compartment, showing statistical differences (B, figure 22). The latency until first entry on the light compartment did not show differences in the 10-minute group, but was decreased in the 45-minute group (C, figure 22).

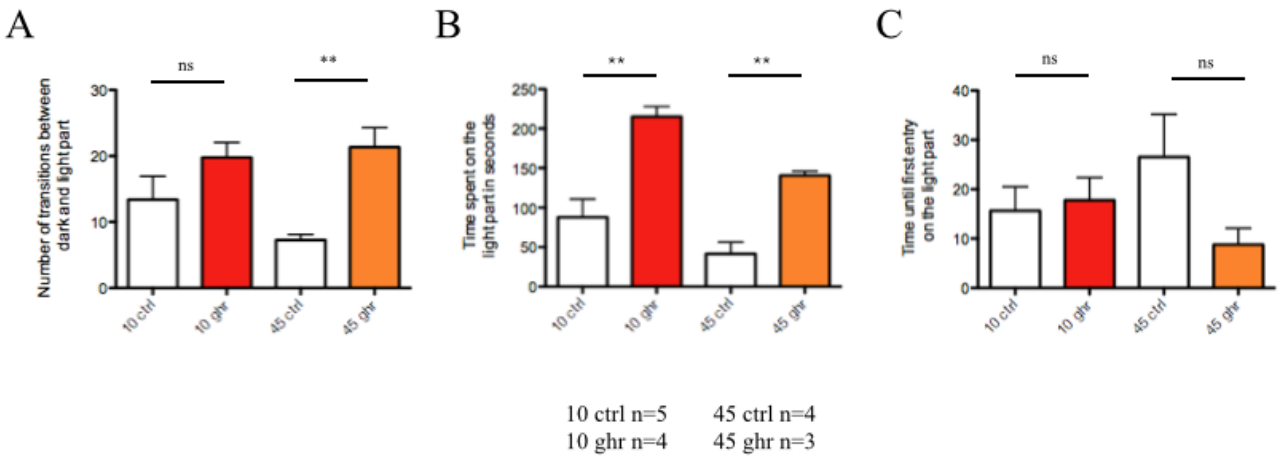


Figure 18 - Effect of ghrelin on anxiety as evaluated by the dark-light box test. (10 Ctrl/Ghr: 10 min condition control and ghrelin treated; 45 Ctrl/Ghr: 45 min condition control and ghrelin treated) **A**: Number of transitions between the dark and light compartments. In both 10 and 45 minutes conditions, ghrelin shows a tendency to increase the number of transitions, although the differences between the groups were not statistically significant. **B**: Time spent in the light compartment. In both 10 and 45 minutes, ghrelin significantly increased the time spent in the light compartment. **C**: Latency until first entry in the light compartment after start of the test. There were no changes in latency for the 10 minutes condition, bu there was a tendency towards a decrease in the latency for the 45 minutes condition. Statistics was done with unpaired t-student test (** $P < 0.01$). Error bars represent s.e.m and n represents the number of independent animals.

Overall, these results suggest that ghrelin decreases anxiety in mice at both 10 and 45 minutes after its i.p. administration. At 10 minutes this effect on anxiety may be overshadowed by a decrease in motor activity, which can be evident on a test heavily reliant on motor activity, such as the open-field test.

4.2.4 MK-0677 decreases anxiety on the open-field test if animals are tested 10 minutes after its administration, has a relatively smaller effect on motor activity in this group in comparison to ghrelin, and shows a tendency to decrease anxiety if animals are tested 45 min after administration

In order to clarify the reason behind the decrease in motor activity and apparent increase in anxiety on the open-field test in animals tested after 10 minutes of ghrelin administration, we repeated the open-field test previously done, administering a potent ghrelin receptor agonist, MK-0677, instead of ghrelin. This agonist has high specificity for the GHS-R1a and has a half-life of >6h, much longer than that of ghrelin (Chang et al., 1996; Jacks et al., 1996). We observed that for the 10 minutes condition, animals injected with ghrelin spent more time in the center area of the open-field, which can be interpreted as a decrease in anxiety (figure 23). At the 45 minutes condition, there is only a slight tendency towards an increased time spent in the center area. In the case of motor activity, there is a slight decrease in the 10 minutes condition, although at a smaller extent than in the 10 minutes condition of animals injected with ghrelin (A, B, figure 23). These tendencies become clearer if we average the sum of the distance and time values for the first 10, 30 and 45 minutes of the test, for each animal in each condition (C,D, figure 23). With the heatmap analysis of the time spent in the different regions of the open-field arena, analogous conclusions can be drawn (figure 24).

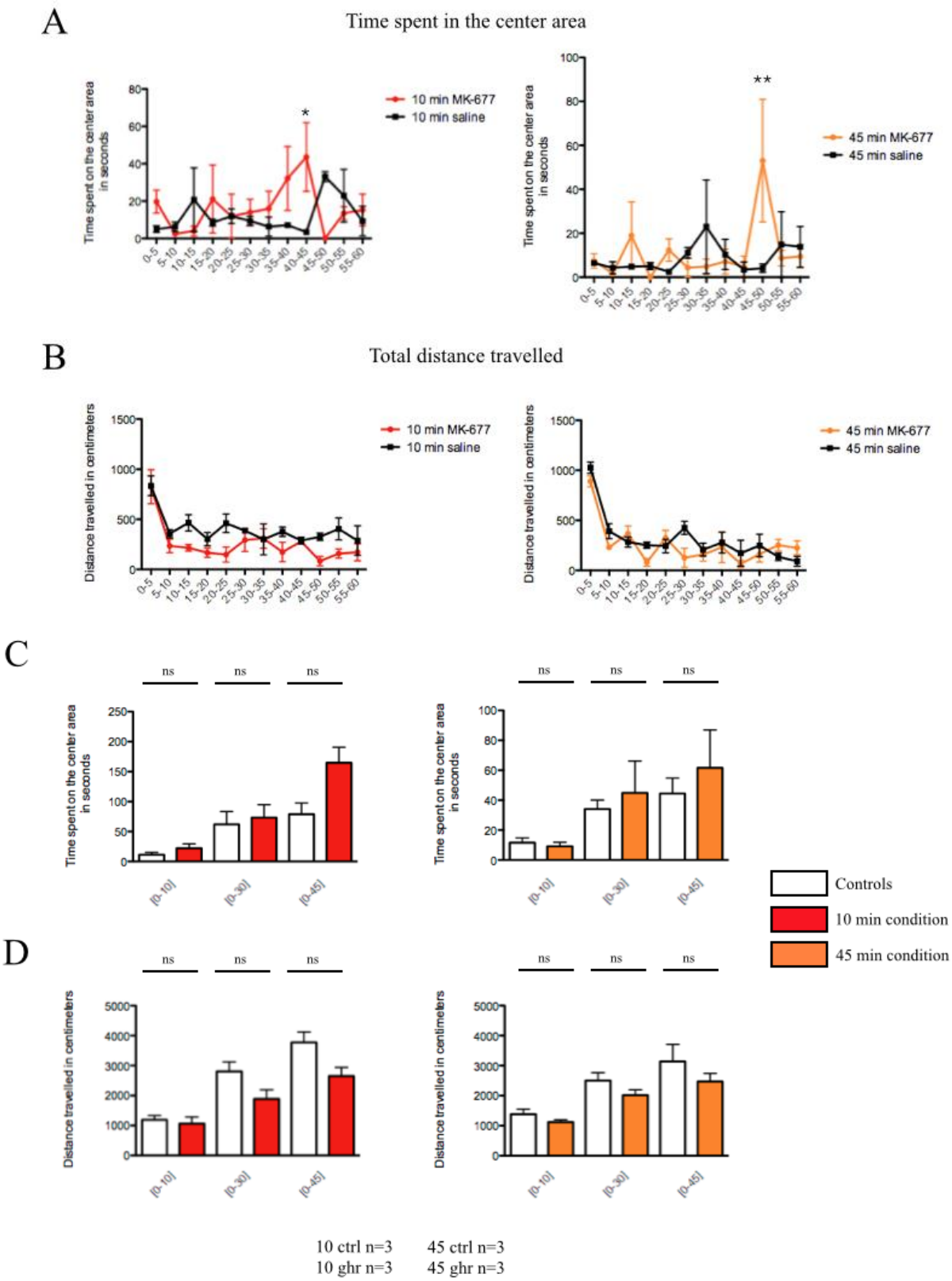


Figure 19 – Effect of MK-0677 on anxiety as evaluated by the open-field test. A,B : Time spent in the center area (A) and total distance traveled (B) on the open-field plotted as average values during increments of 5 minutes for the duration of the test (60 minutes). For the 10 and 45 minutes conditions, animals injected with MK-

0667 have a tendency to spend more time in the center area. Two-way ANOVA test did not show statistical differences between treatments, but Bonferroni *post hoc* test showed a significant increase in the time spent on the center area for the 40-45 minutes increments for the 10 minutes condition and 45-50 minutes increment for the 45 minutes condition (* $P < 0.05$; ** $P < 0.01$). There is a tendency towards a decreased motor activity for the 10 minutes contidion. **C,D**: Time spent in the center area (C) and total distance traveled (D) on the open-field presented as average of the sum of the values for the first 10, 30 and 45 minutes, for each animal in each condition. The observed increase in time spent in the center and lower decrease in motor activity for the 10 minutes condition are more evident. Unpaired t-student test did not show statistical differences between the groups. For the whole panel error bars represent s.e.m and n represents the number of independent animals.

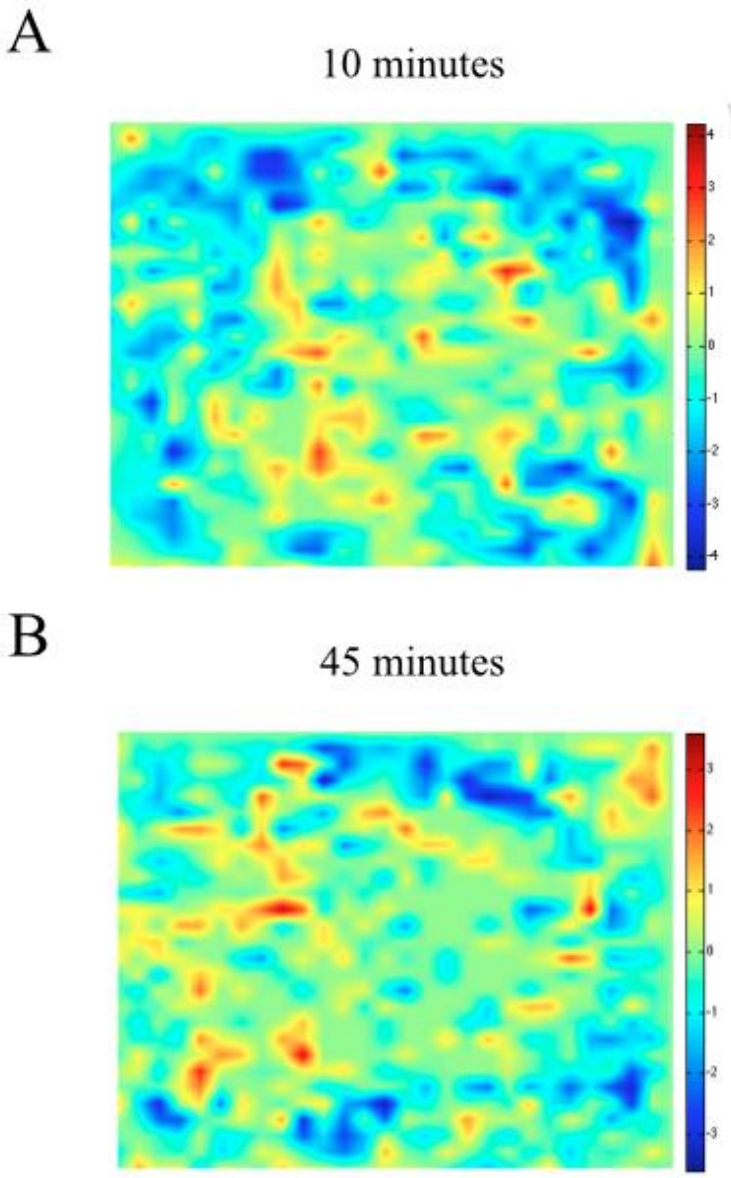


Figure 20 - Heatmaps of the open-field test for MK-0677 and saline treated animals. The XY position of mice overtime was converted into the frequency of the presence of the animals on a two-dimensional matrix representing the open-field arena. These matrices were summed for all the animals in each condition and the values were converted into the logarithmic scale. The matrix for the control conditions was subsequently substracted from the ghrelin conditions: values above 0 represent areas where animals treated with ghrelin were more present and values below 0 represent areas where animals treated with ghrelin were more absent, in comparison to control. **A** represents the 10 minute conditions and **B** represents the 45 minute conditions. These heatmaps indicate that animals treated with MK-0677, both 10 minutes and 45 minutes groups spent slightly more time in the center than controls, although at the 45 minutes this difference was less pronounced. n=3 for all conditions.

These results suggest that the stimulation of the GHS-R1a receptor, with ghrelin or MK-0677, leads to a decrease in anxiety observed at 10 and 45 minutes.

4.3 SUPPLEMENTARY RESULTS

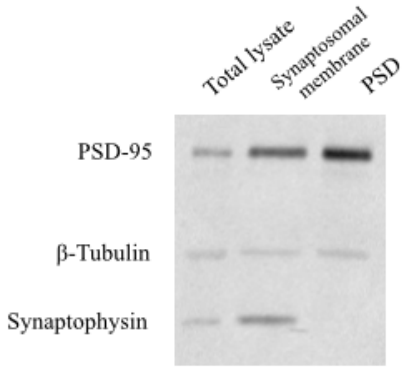


Figure 21 - Example of quality control blot for the PSD purification technique. Purification is represented by increased levels of PSD-95, decreased Synaptophysin and comparable level of Tubulin from brain lysate towards the more pure PSD fraction. Quality control western blots were performed for every conditions of every replicate in the ghrelin removal experiment.

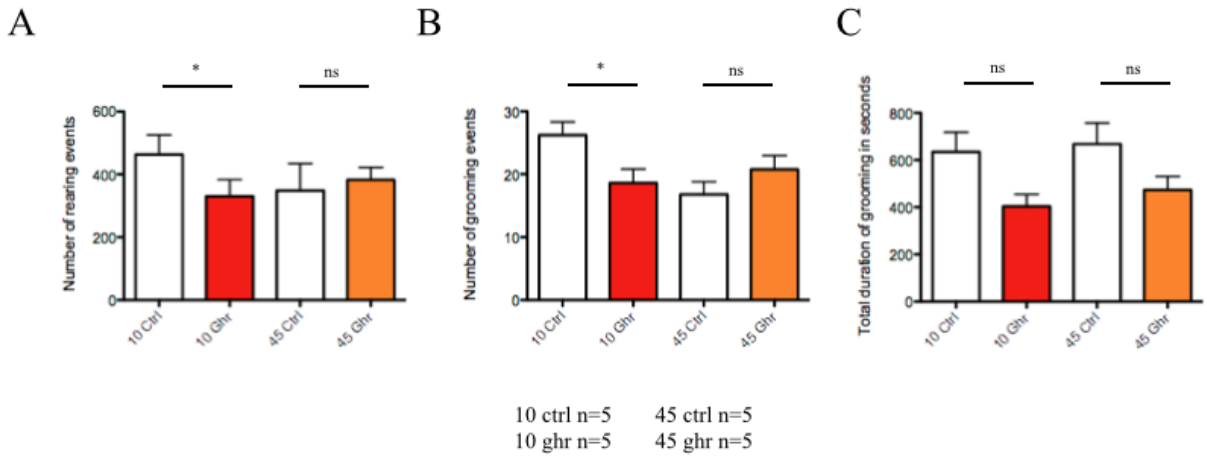


Figure 22 – Number of rearing events (A) and number (B) and total duration (C) of for the second cohort of animals tested on open-field with ghrelin. (10 Ctrl/Ghr: 10 min condition control and ghrelin treated; 45 Ctrl/Ghr: 45 min condition control and ghrelin treated) Remarkably grooming number and duration is significantly decreased in the case of the 10 min ghrelin-treated group, which can be interpreted as a decrease in anxiety. This anxiety-related parameter of the test may not be so much affected by the observed decrease of motor activity in this experimental group and thus already show the decrease in anxiety observed on other behavioral tests for this experimental group. Unpaired t-student test showed statistical differences between several groups (* $P < 0.05$) Error bars represent s.e.m and n represents the number of animals.

Table 2 – Weights of the animals for each condition and each test used, expressed in average grams ± s.e.m.

	10 min Ctrl	10 min Ghr	45 min Ctrl	45 min Ghr
Open-field with Ghrelin	25.3±0.4	26.0±0.5	26.0±0.5	26.2±0.5
Open-field with MK-0677	25.1±0.7	28.3±1.9	24.4±0.6	25.5±0.2
Elevated-Plus Maze	26.8±1.6	28.6±0.7	28.2±1.5	27.1±1.2
Dark-Light Box	28.7±1.4	28.9±0.8	28.5±0.5	30.4±0.2

Chapter 4 – Discussion and Conclusion

5 DISCUSSION

At a behavioral level, there is consensus in the literature that ghrelin regulates hippocampal-dependent memory (Moran and Gao, 2006). At the synaptic level, the molecular mechanism at the basis of this effect on memory has begun to be elucidated by the work of Luís Ribeiro (Ribeiro et al., 2014). The activation of the GHSR1a is responsible for an increase of cell surface and synaptic GluA1 receptors and a decrease in the threshold for LTP. These effects required activation of the ghrelin receptor by MK-0677 for 1 and 20h respectively. While these observations represent a critical part of our understanding on how ghrelin may mediate memory formation, at the physiological level they represent only one side of what the mechanism for ghrelin-mediated memory-enhancement might be.

The levels of plasmatic ghrelin oscillate throughout the day in a manner regulated mainly by feeding (Cummings et al., 2001a). Plasmatic ghrelin increases two-fold immediately before each meal and decreases to trough levels within 60-120 minutes after food intake. This means that each increase in ghrelin is accompanied by a decrease, of similar magnitude. We may then speculate that the dramatic effect on the hippocampal excitatory synapse molecular composition that occurs when ghrelin is increased, may be paralleled by changes in the hippocampal synapse molecular composition when ghrelin levels decrease, as happens after each meal. This was the fundamental hypothesis that our experiments tried to explore. We found that the abrupt decreases of ghrelin levels have also a marked effect on hippocampal glutamatergic synapses molecular composition. This knowledge, together with the previous findings by Luis Ribeiro and Fiona van Leewuen, provide a more integrative view of how ghrelin modulates synaptic transmission in the hippocampus.

Until now, neither the effects of ghrelin on anxiety nor the mechanism behind these effects have been clearly addressed (Andrews, 2011). Ghrelin seems to both increase and decrease anxiety in different paradigms and across different animal models. In the literature there is a clear lack of experiments that try to integrate both effects on anxiety within the same paradigm and animal model. The most promising hypothesis to explain this elusive effect on anxiety is that the interval of time between ghrelin administration and the start of the behavioral test could have an impact on the changes on anxiety induced by ghrelin. We set out to test this hypothesis but our results suggest that this explanation may be false. Our observations indicate that ghrelin administration leads to a decrease in anxiety on two out of three behavioral tests. In the third test (open-field) 10 minutes following ghrelin administration the animals show a decrease in motor activity. This observation may confound results regarding anxiety in this test in particular. With these results we are beginning to understand how ghrelin may modulate anxiety-like behavior. Additionally, these provide us a platform to understand the cognitive effects of ghrelin that may be indirectly affected by anxiety.

5.1 ABRUPT DECREASES OF GHRELIN AND THEIR PHYSIOLOGICAL RELEVANCE

With our ghrelin removal protocol we tried to reproduce the physiological context of the abrupt decreases of ghrelin seen *in vivo*. We gave preference to using hippocampal organotypic slice cultures since they conserve the canonical hippocampal tri-synaptic circuit. It was observed by Luís Ribeiro that PKC was most highly activated by GHS-R1a after 5h of stimulation with MK-0677. PKC is one of the most important pathways in GHS-R1a signaling (Luís Ribeiro PhD thesis, unpublished work). Thus, this time point can be interpreted as the period of stimulation of GHS-R1a that achieves the maximal intracellular signaling. We chose 30 minutes before the end of these 5h as the timepoint for removal of ghrelin, in order to reproduce the half-life of the hormone (~30 minutes) (Akamizu et al., 2004).

Stimulation of hippocampal neurons for 5h with ghrelin did not produce a statistically significant increase in the levels of GluA1 in post-synaptic densities. Nevertheless, there was a trend towards an increase in overall levels of GluA1 following ghrelin stimulation as assessed in hippocampal lysates. This would suggest that overall level of GluA1 increase with ghrelin signaling, but their insertion into PSD slots may require further stimulation. It was observed that chemical LTP increases robustly the synaptic levels of GluA1 when neurons are treated for 1h with MK-0677, as evaluated by immunocytochemistry. This increase was of higher magnitude than the increase observed with just MK-0677 (Ribeiro et al., 2014). It should be noted that our experiments are the first to look directly at the effect of ghrelin on post-synaptic density molecular composition.

In contrast with the above, we observed a robust decrease in the levels of synaptic GluA1 following the abrupt sequestration of ghrelin. This is a previously undescribed observation and was consistent in 7 experimental replicates. A decrease of this magnitude may indicate that after a certain period of activation of GHS-R1a, the absence of ghrelin, and thus the ceasing of GHS-R1a activation, leads to a decrease on the levels of synaptic GluA1 on a majority of hippocampal glutamatergic synapses. We also showed that the GluA1 receptors removed from post-synaptic densities may be most likely degraded briefly after the removal. In order to test whether this degradation of GluA1 significantly affected the overall levels of GluA1 at the cell surface, it would be interesting to perform biotinylation assays targeting the GluA1 subunit. The increase in GluA1 expression at the whole cell level after 5h of ghrelin stimulation, in conditions similar to ours, has also never been reported.

We did not observe any changes on the levels of NMDA receptor subunit GluN1, neither after 5h of stimulation nor with abrupt removal of ghrelin. This helps support the idea that the effects of ghrelin at a synaptic level may only be directed at specific molecules. To give further evidence to this hypothesis, tests can be run for other molecules, such as NR2 receptors and scaffolding molecules such as Shank or Homer molecules. In the case of PSD-95, one of the main scaffolding proteins of the post-synaptic density, the removal of ghrelin or the 5h stimulus did not observably change its levels at the post-synaptic density. However, ghrelin stimulation, regardless of it being removed 30 minutes before the 5h or not, increased the levels of PSD-95 expressed at the whole cell level. This corroborates the observation by Fiona van Leeuwen that, in primary hippocampal neuron cultures, levels of PSD-95

clusters are increased after 1h stimulation with MK-0667, as assessed by immunocytochemistry. This observation may be related with the finding that GHS-R1a agonist-induced activity promotes spine maturation on mature hippocampal neurons (unpublished work, Fiona van Leeuwen Masters Thesis). The assessment of the effects of GHSR1a activation on PSD-95 under our conditions may require additional experimental replicates.

In terms of what the intracellular mechanism of signaling of the ghrelin removal/GluA1 decrease phenomenon could be, we can put forward various hypotheses. Recent work by Luís Ribeiro identified that after 20h stimulation with the inverse agonist of GHS-R1a , hippocampal neurons show a decrease of GluA1 puncta, as assessed by immunocytochemistry (unpublished work). The magnitude of this decrease was similar to the magnitude of the decrease observed after the removal of ghrelin. The inverse agonist of GHS-R1a has the effect of antagonizing not only the ghrelin-induced activity of GHS-R1a but also inhibiting the constitutive activity of the receptor. We may speculate that after the sequestration of ghrelin, GHS-R1a could lose not only its ghrelin-induced activity but also its constitutive activity. This hypothesis could be tested either by the application of surplus quantities of the inverse agonist at the same time point as ghrelin removal but without alteration of the media levels of ghrelin, or attempting to occlude this effect with the inverse agonist by its application before the ghrelin removal and in this case removing ghrelin from the media at the previously tested time point. It should be noted that simply stimulating primary neuron cultures with GHS-R1a antagonist for 1h does not alter the synaptic expression of GluA1, as assessed by immunocytochemistry (Ribeiro et al., 2014). An already described physiological condition in which GHS-R1a stops signaling, including constitutive activity, is through a processes of desensitization. It was observed that following activation of the GHS-R1a by ghrelin, the GHS-R1a is phosphorylated and β-arrestin 2 is recruited to the receptor which then signals the receptor for internalization and degradation or recycling (Mear et al., 2013). This desensitization of GHS-R1a happened at ~20 min after exposure to ghrelin in HEK cells tranfected with GHS-R1a, as assessed by radioligand binding studies and confocal miscroscopy (Camina et al., 2004). The dynamics of GHS-R1a desensitization have not yet been evaluated in neurons. We thus can hypothesize that a mechanism involving the loss of constitutive activity of GHS-R1a may be responsible for the ghrelin removal/GluA1 decrease phenomenon. In order to test this hypothesis the neuronal membrane and intracellular levels of GHS-R1a could be evaluated through immunocytochemistry or biotinylation assay during a time course of ghrelin stimulation, or the intracellular signaling activity of the receptor could be evaluated through calcium imaging in neurons, since activation of the receptor is known to mobilize intracellular stores of calcium (Camina et al., 2004).

It should also be informative to study how this phenomenon is explained by intracellular signaling mechanisms. GHSR1a activation leads to the activation of the PKA pathway (which leads to the phosphorylation of Ser^{845} in GluA, a critical step towards synaptic incorporation), and the activation of PI3K and PKC (Ribeiro et al., 2014). This suggests that GHSR1a can couple itself to various types of G-proteins (mainly to $G_{\alpha q/11}$ but also to G_q and $G_{i/o}$) which indeed has been already demonstrated (Camina,

2006; Howard et al., 1996; Smith et al., 2007). These signaling pathways along with MAPK are involved in GluA1 priming and LTP, which at the post-synaptic density level is manifested by an increase in the levels of GluA1 receptor (Luscher and Malenka, 2012). Therefore we would expect these pathways to be inhibited. On the other hand, the downscaling process of homeostatic plasticity constitutes a synaptic plasticity mechanism that manifests itself in a manner similar to our phenomenon: both showing a generalized decrease in the AMPA receptor synaptic levels (Wang et al., 2012). Of particular interest is the work of Hou and colleagues (Hou et al., 2011). They observed that by light-mediated increase in pre-synaptic terminal activation, hippocampal neurons in culture showed a selective reduction on the abundance of AMPA receptors on stimulated synapses, thus establishing a paradigm for homeostatic downscaling of single synapse activity. This process was independent of calcineurin or NMDA-NR2B-dependent signaling but it was accompanied by enhanced AMPA receptor ubiquitination and degradation. The degradation process observed was remarkable in that it happened locally in dendrites or spines independently of the soma and within just 30 minutes of light stimulation. It is conceivable that the signaling involved in our phenomenon may use the same pathways as an homeostatic plasticity mechanism of downscaling. We observed a degradation of AMPA receptors within 30 minutes of ghrelin removal, but it is not clear whether this happens just on synapses expressing GHSR1a or at a global level. Either way it would be relevant to consider the dependence of our phenomenon on the ubiquitination/proteasome system, which could be tested by performing the ghrelin removal protocol in the presence of proteasome inhibitors.

If we take the findings from the laboratory of Ana Luísa Carvalho and integrate them with the ghrelin removal/GluAl decrease findings described in this thesis, we arrive at a more physiologically relevant hypothesis for how ghrelin may modulate neuronal circuitry and behavior. Throughout the day there are peaks and troughs in ghrelin levels (Cummings et al., 2001a). Activation of GHSR1a primes AMPA receptors for synaptic delivery and lowers the threshold for LTP induction (Ribeiro et al., 2014). It can be assumed that ghrelin, signaling mainly through GHSR1a, can mediate these effects following changes in physiological levels. This would mean that hippocampal excitatory synapses that happen to be activated during peaks of ghrelin would more easily be potentiated, with increases in AMPA receptor content on synaptic membranes. These peaks of ghrelin coincide with the period at which an animal would be engaged in searching for food. At this point, enhanced memory formation is particularly useful for the animal, to later recollect the food location and conditions. Spatial memory traces have been described to correspond to LTP-induced maps of potentiated synapses on the hippocampus (Dragoi et al., 2003; Whitlock et al., 2006). Thus, ghrelin could provide a boost on the formation of these maps. After feeding, levels of ghrelin decrease to trough levels and according to our observations, the decrease in ghrelin levels would lead to a robust decrease of GluA1 synaptic levels. We can hypothesize that synapses that are potentiated during the ghrelin peak will remain relativeltly potentiated in comparison with the remainder of non-potentiated synapses, if this decrease happens in a linear and homogenious manner along the majority of the hippocampal glutamatergic synapses. If we take into account the observation that NMDA receptor levels are unchanged, the decrease of GluA1 levels on unpotentiated

synapses may even result in the formation of silent synapses, which are synapses unable to be stimulated due to the abundance of NMDAR receptors (which are only capable of activation after membrane depolarization) and absence of AMPA receptors. Even without the decrease in all AMPA receptor types, the decrease in GluA1 synaptic levels would result in an increased contrast between the population of unstimulated synapses and stimulated/potentiated synapses. At the circuit level, this would increase the signal to noise ratio of memory traces coded by patterns of potentiated synapses; patterns which in turn were generated during the discrete time interval of high ghrelin levels. A similar mechanism was recently observed for the action of glucocorticoids on mouse cortex related to learning, by Liston and coworkers (Liston et al., 2013): circadian glucocorticoid peaks promote post-synaptic dendritic spine formation on the cortex after motor skill learning, whereas troughs are required to stabilize newly formed spines important for long-term memory retention. Chronic exposure to glucocorticoids eliminates learning-associated new spines and disrupts the previously acquired memories.

This is an interesting hypothesis for an undescribed mechanism by which a particular physiological state, in this case hunger, signaling through a particular hormone, ghrelin, is capable of modulating in a comprehensive manner the process synaptic strenght. This hypothesis can be tested by a series of experiments. First, the ghrelin removal/GluA1 decrease phenomenon should be reproduced in primary hippocampal neuron cultures. Next, using sparse primary hippocampal neuron cultures, we can sparsely transfect neurons with ChR2-GFP constructs, a light-activatable ion channel capable of membrane depolarization. Then we couple the ghrelin removal protocol with optically-induced stimulation of presynaptic neurons and selective simulate the potentiation of hippocampal synapses. We can then evaluate whether the sequestration of ghrelin decrease in GluA1 is confined to synapses contacting presynaptically with non-stimulated neurons or is also observed on synapses contacting presynaptically with light-stimulated neurons. The aforementioned questions on the mechanisms of this decrease in GluA1 can also be more clearly addressed on this paradigm.

5.2 GHRELIN EFFECT ON ANXIETY AND ITS ROLE ON GHRELIN-MEDIATED MEMORY ENHANCEMENT

Ghrelin modulation of anxiety is an elusive topic of research. In our experiments, we tested the hypothesis that ghrelin affects anxiety in a time-dependent manner: during an initial period of ~10 minutes we predicted that it should induce an increase in anxiety and after a period of ~45 minutes it should provoke a decrease in anxiety. In order to test this hypothesis we used 3 widely studied behavioral anxiety tests: the open-field test, the elevated-plus maze test and the light-dark box test. We chose to use mice as our animal model because this was the model used on several of the seminal work regarding the effects of ghrelin on memory and anxiety (Carlini et al., 2008; Lutter et al., 2008).

Regarding the anxiety levels 10 minutes after administration of ghrelin, the behavioral tests performed were mostly consistant towards ghrelin decreasing anxiety. This was most evident on the elevated-plus maze and light-dark box test. This observation contradicts the majority of results reported in the literature, which follow the seminal experiments by Carlini and coworkers (Carlini et al., 2002b) and Asakawa and coworkers (Asakawa et al., 2001). In the case of Carlini and coworkers, the open-field and elevated-plus maze tests were ran 10 minutes after intracerebral injection of ghrelin (at 0.3 nmol, 1.5 nmol and 3 nmol) to the hippocampus. In this case Wistar rats were used and 10 to 6 animals were used per condition. The open-field test showed that ghrelin did not alter the time spent in the central area but, for 0.3 nmol, it significantly increased the locomotion of the animals and, for 1.5 nmol and 3 nmol, increased the freezing of the animals. The increased freezing of the animals here observed is consistent with our observation of decreased locomotor activity, although in this study it did not affect the time spent in the center area. It should be highlighted that the open-field test in this study was only ran for 10 minutes, so changes in locomotor activity could become more evident if the test was run for a longer period. In the case of the elevated-plus maze, ghrelin (at 1.5 nmol and 3 nmol) significantly decreased the number and percentage of entries on the open arms, and the percentage of time spent on the open arms. No significant differences for total number of entries were reported. The experiments conducted on this study have been recently replicated by Kajbaf and coworkers, for overectomized female rats and with the ghrelin receptor agonist GHPR-2, obtaining similar results (Kajbaf et al., 2012). In the case of Asakawa and coworkers, the elevated-plus maze test was run 10 minutes after icv injection of ghrelin (at 0.03nmol, 0.3 nmol, 1 nmol and 3 nmol). In this case male ddy mice were used, with 7 weeks of age, and 10-13 animals were used per condition. The elevated-plus maze dimensions were similar to ours. The test showed a consistent decrease on the percentage of time and entries in the open arms for 0.3 nmol, 1 nmol and 3 nmol. The total number of entries was not affected. The test was only run for 5 minutes.

Perhaps the most evident differences between these two studies and ours lay on the route of delivery of ghrelin. Peripheral murine ghrelin does not cross the blood-brain barrier, but it acts on certain brain regions with a more permeable contact between neurons and circulation, namely the hypothalamus, the subfornical organ (Pulman et al., 2006) and area postrema (Banks et al., 2002; Diano et al., 2006; Fry and Ferguson, 2010). Intra-peritoneal or subcutaneous injections as a way to deliver ghrelin to the central

nervous system are thus not only convenient but provide a more physiologically relevant delivery of the hormone. These routes of delivery ensure that, in principle, the brain circuits that are physiologically regulated by ghrelin, receive it and contribute for the modulation of behavior. Although experiments conducted in this manner will not reveal the specific contribution of a certain brain region for the action of ghrelin on behavior, they will reveal behavioral features more proximate to what results from high level of ghrelin in physiologic conditions and will also be able to suggest the potential pharmacological application of the hormone through less invasive administration routes. Indeed, one of the most recent major studies regarding the effects of ghrelin on anxiety administered ghrelin peripherically (Lutter et al., 2008). This was also the study that tested animals 45 minutes after administration of ghrelin, and showed a decrease in anxiety. Lutter and coworkers used the elevated plus-maze test, ran for 5 minutes, after subcutaneous injections of ghrelin (at 2 μ g or 0.6 pmol per g of animal weight). 8 animals per condition were used. Ghrelin significantly increased the time on open arms, the distance traveled on open arms and the percentage of entries on the open arms. This is in agreement with our observations on the elevated-plus maze and the dark-light box. To further reinforce our observations, more animals should be run on the elevated-plus maze test and light-dark box. It remains possible that there may exist an unidentified circuit by which ghrelin secreting neurons, within brain regions involved on anxiety, would be involved in the fine-tuning of this behavior, either enhancing or inhibiting it. If so, ghrelin directly injected into regions like amygdala (Alvarez-Crespo et al., 2012; Currie et al., 2014) or discrete hypothalamic nuclei (Currie et al., 2012) could be activating effector neuron populations on this circuit, influencing anxiety-like behavior in an heterogeneous manner depending on the activated part of the circuit or the context of the organism.

Overall we observed that ghrelin decreases anxiety, although this is not evident for the 10 minutes condition on the open-field. We propose that the main reason behind this is a decrease in motor activity. To further elucidate this phenomenon we conducted the same test but this time administering MK-0677, a highly specific agonist of the GHS-R1a, with an half-life much longer than ghrelin (>6 hours compared to ~30 minutes, respectively). We observed that at the 10 minutes condition there was a decrease in anxiety, as assessed by an increase in the time spent in the center area, and only a slight decrease in the motor activity. It may be the case that the effect of ghrelin decreasing motor activity in the 10 minutes condition is signaled by a different receptor from GHS-R1a. The presence of a specific uncharacterized ghrelin receptor has already been described in chondrocytes (Caminos et al., 2005), human erythroleukaemic HEL cells (De Vriese et al., 2005), and cardiomyocytes (Iglesias et al., 2004).

Probably the most physiologic manner to evaluate the effects of ghrelin on anxiety is to induce increases in endogenous ghrelin secretion, which is possible through repeated water stress (Meyer et al., 2013), chronic social defeat and caloric restriction (Lutter et al., 2008). Afterwards the anxiety could be tested. An important difference between ghrelin and MK-0677 is the fact that mouse ghrelin, which diverges from rat ghrelin only in two aminoacids, has not been shown to readily cross the blood brain barrier (Fry and Ferguson, 2010), while MK-0677 does cross the blood brain barrier (Meyer et al., 2013). This means

that the observed effects of ghrelin are the result of its actions on the aforementioned regions lacking a normal blood-brain barrier, while the observed effects of MK-0677 results from its overall action on brain regions were GHS-R1a is present. Ultimately, our experiments point towards the hypothesis that an anxiety decreasing effect of the activation of the GHSR1a receptor may be overshadowed by a decreased motility at least soon after ghrelin administration. To test this hypothesis we could administer an GHSR1a specific antagonist simultaneously to rat ghrelin and conduct the same behavioral tests. We cannot disregard the fact that high concentrations of ghrelin, as the ones achieved by our injections, may give rise to behavioral changes not representative of the effects of physiologic peak levels ghrelin; therefore experiments should be performed to elucidate this fact.

The observation that ghrelin decreases anxiety helps the understanding of how ghrelin increases memory. In humans, anxiety reduces working memory, by perturbing attention and giving more relevance to information that appears to be threatening over other potentially important information (Vytal et al., 2013). Thus reducing anxiety, may enable the animal to process information more efficiently and form more stable memory traces. This is advantageous in a wild environment, where animals would see their baseline for anxiety decrease due to the need for exploring new environments in search of food.

A recent and remarkable study by Meyer and coworkers unveiled a function for ghrelin on stress-induced vulnerability to fear, a phenomenon at the basis of depression, anxiety and posttraumatic stress disorder (Meyer et al., 2013). This study used rats that were repeatedly exposed to a stressor and afterwards displayed increased fear learning following Pavlovian fear conditioning. The stressor increased circulating ghrelin, which was necessary and sufficient for the stress-associated vulnerability to exacerbated fear learning, these actions taking place at the amygdala and requiring ghrelin-mediated release of growth hormone. This may mean that increases in memory and decreases in anxiety mediated by ghrelin may be related with the acquisition of maladaptive memories related with, for instance, PTSD. There may appear to be a paradox, since PTSD is a disorder of increased anxiety, but these observations can be reconciled by considering that the decrease in anxiety may be transient, taking place during the acquisition of the maladaptive memory (in the case of PTSD this will correspond to the traumatic event), and the retrieval of memories may not be directly related with ghrelin (Carlini et al., 2010). The work by Meyer and coworkers also showed that injecting MK-0677 for 5 consecutive days and testing animal 24h after the last injection, there were no changes in anxiety, as assessed by the elevated-plus maze (Meyer et al., 2013), thus suggesting that the effect that we observed after ghrelin administration may be transient.

5.3 GHRELIN IN MEMORY AND STRESS

Ghrelin plays an important role on learning and memory formation. Caloric restriction, decreases age-related deficiencies in cognitive processes (Witte et al., 2009), enhances learning and facilitates synaptic plasticity (Fontan-Lozano et al., 2007). Ghrelin may be related with these benefits, since it is increased during caloric restriction (Lutter et al., 2008). Decreases in ghrelin may also be related to impaired

cognition, since high fat and glucose diets, which reduce ghrelin secretion (Lomenick et al., 2009), impair hippocampal synaptic plasticity and spatial memory (Stranahan et al., 2008). In this thesis we began to uncover a mechanism by which ghrelin, through its oscillating levels, may change glutamate receptor synaptic composition. Ghrelin is involved in stress signaling, as it is suggested by the increases in the levels of ghrelin after induction of different types of stress. Indeed, stress is an external or internal signal indicating the potential or perceived threat (Maras and Baram, 2012). The necessity of feeding signaled by ghrelin is an internal type of stress that requires certain adaptive processes to be engaged in order to maintain homeostasis. The hippocampus is particularly vulnerable to the effects of stress (Joels and Baram, 2009): during mild or short-lasting stress, the hippocampal function is often enhanced by augmentation of synaptic plasticity, which may reflect the adaptive importance of remembering threatening circumstances and how to deal with them (Schwabe et al., 2012). This is in agreement with the described functions of ghrelin and with our observations: ghrelin may be seen as a signaling molecule for a very particular type of stress that at a short-term decreases anxiety, an important component of stress response. However, when these mechanisms are activated intensely or for a prolonged period, the hippocampus may become susceptible to the detrimental effects of chronic or severe stress. Chronic stress impairs learning and memory in both humans and experimental animals (Schwabe et al., 2012). Indeed, a detrimental effects of ghrelin chronically elevated levels of ghrelin have already been shown (Meyer et al., 2013), relating ghrelin with anxiety disorders. Our work, and these previous observations, point towards ghrelin being a key player on stress responses and on stress-related pathologies.

6 CONCLUSION

In this thesis work we have shown that abrupt decreases of ghrelin robustly reduce the levels of GluA1 on hippocampal glutamatergic synapses, without affecting PSD-95 and GluN1 content. We have also shown that the activation of the GHSR1a receptor decreases anxiety independently of the time between its administration and the start of the behavioral tests. We also demonstrated that ghrelin decreases motor activity 10 minutes after its administration which, together with the fact that this is not observed if a specific GHSR1a agonist is administered, may point towards the existence of other receptors through which ghrelin modulates behavior. These findings are important to understand the molecular basis of ghrelin effects on cognition and for future pharmacological applications of the modulation of the ghrelin system. They are also important for the understanding of anxiety-related disorders.

Ghrelin is an unique peptide hormone, in that it establishes a link between food intake and cognitive functions. It acts on a wide variety of brain regions and the full extent of its actions still remains to be unraveled. There are clear benefits in seeking an explanatory model for the functions of ghrelin on cognition, in that its effects become founded on molecular and cellular mechanisms. This knowledge may provide the foundation for the development of therapeutic strategies for metabolic related disorders, such as obesity and diabetes, neurodegenerative disorders, such as Parkinson's or Alzheimer's disease,

and psychiatric disorders, such as post-traumatic stress disorder. The understanding of the effects of the ghrelin system on cognition is thus of outstanding social and economical importance.

Chapter 5 – References

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