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Peptides that signal hunger – a new role in memory and social behaviors

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Abstract

Neuroactive peptides and their receptors in neurons are a large group of molecules essential for central nervous system homeostasis. They participate in the control of basic functions, such as regulation of the cardiovascular system and gastrointestinal tract, and in higher function, such as cognition and social behavior. In this work we provide an overview of common features of these signaling molecules, their receptors and explore how they regulate two important behaviors: memory and social behaviors. We describe the role of the constitutive activity of the ghrelin receptor on learning and memory and explore a mechanism for its effect on neuronal transmission. Through pharmacologic manipulation, we observed that the ligand-independent activity of the ghrelin receptor regulates the surface and synaptic expression of AMPA receptors on neurons. This effect is exerted through the regulation of AMPA receptor mobility and phosphorylation state. On another section of the work, we describe alterations to the prefrontal cortex in a model of early life stress. We find that social subordinate behavior is induced by stress and explore the mechanism leading to this behavioral alteration. We find increased inhibition in pyramidal neurons of the prefrontal cortex in subordinate animals accompanied by increased expression of the Y_1 receptor for neuropeptide Y. Using whole-cell patch-clamp recordings, we observed a reversion of the increased inhibition in the prefrontal cortex of subordinate animals when using an NPY Y_1 antagonist. In addition to its well-known role in feeding behavior, our observations suggest that the neuropeptide Y signaling plays a role in the processing of social behaviors in the mammalian brains. This work illustrates the versatility of neuroactive peptides in the regulation of animal behavior. We provide potential avenues for the development of treatments for diseases associated with cognitive deficits and dysfunctional subordinate behavior.

Keywords: Neuropeptides, Ghrelin receptor, Constitutive activity, Social dominance hierarchies, Neuropeptide Y

Resumo

Os neuropéptidos e seus recetors constitutem um vasto grupo de moléculas essenciais para o controlo da homeostasia por parte do sistema nervoso central. Estas moléculas participam no controlo de funções básicas, como regulação do sistema cardiovascular e do trato digestivo, e funções complexas, como a cognição ou comportamento social. Neste trabalho resumimos as características comuns dos neuropéptidos e seus recetores e exploramos como regulam dois importantes aspetos do comportamento animal: memória e hierarquias de dominância social. Neste trabalho decrevemos o papel da atividade constitutiva do recetor da grelina para a memória e exploramos um mecanismo que poderá dar origem a este efeito. Através de manipulações farmacológicas, verificámos que este tipo de atividade regula a expressão superficial e sinática de recetores AMPA em neurónios. Este efeito decorre da regulação da mobilidade dos recetores, de alterações na sua plasticidade sinática e de alterações no estado de fosforilação dos mesmos. Na secção seguinte, e usando um modelo de stress no início da vida, descrevemos novas alterações na neurofisiologia do córtex prefrontal associadas à subordinação social. *In vitro,* verificámos aumento nas correntes inibitórias de neurónios do córtex prefrontal de animais subordinados. O fenótipo de subordinação foi acompanhado de aumentos na expressão do recetor Y_1 para o neuropéptido Y no córtex prefrontal. Através de antagonismo do recetor, observámos uma reversão do aumento na correntes inibitórias no córtex prefrontal destes animais. Estes dados sugerem que o neuropéptido Y tem um papel importante no processamento de informação relativa às hierarquias de dominância social em cérebros de mamíferos. Este trabalho exemplifica a versatilidade dos neuropéptidos e da sua farmacologia na regulação do comportamento animal. Este trabalho abre novas vias para o desenvolvimento de tratamentos para patologias associadas a défices cognitivos e subordinação social disfuncional.

Palavras-Chave: Neuropéptidos, Recetor da grelina, Atividade constitutiva, Hierarquias de dominância social, Neuropéptido Y

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Abbreviations

cAMP	Cyclic adenosine monophosphate
5-HT	5-hydroxytryptamine
Ach	acetylcholine
ACTH	adrenocorticotrophin hormone
ADF	actin-depolymerizing factor
AgRP	agouti-related peptide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
AMPK	AMP activated protein kinase
Arc	arcuate nucleus
ATP	adenosine triphosphate
AZ	AZ12861903 inverse agonist of the ghrelin receptor
α-MSH	α-melanocyte stimulating hormone
BBB	blood brain barrier
BDNF	brain-derived neutrophic factor
BLA	basolateral amygdala
CA1	Cornu Ammonis
CAMK	Ca2+/calmodulin-dependent protein kinase
CART	cocaine and amphetamine responsive transcript
CCk	cholecystokinin
CGRP	calcitnin gene-related peptide
cLTP	chemical longterm potentiation
CRF	corticotropin releasing factor
CRH	Corticotropin releasing hormone
DA	dopamine
DIV	days in vitro
dmPFC	dorsal medial prefrontal cortex
DRD	dopamine receptor
DYN	dynorphin
ELS	early life stress
ENK	enkephalin
EPSC	excitatory postsynaptic currents
EPSP	excitatory postsynaptic potentials
ERK	extracellular signal-regulated kinases
GABA	gamma-aminobutyric acid
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GHSR	growth hormone secretegogue receptor
GnRH	Gonadotropin releasing hormone
GOAT	ghrelin O-acyltransferase

GPCR	g protein-coupled receptor
GTP	guanosine triphosphate
HFS	high frequency stimulation
HPA	hypothalamic-pituitary-adrenal axis
ICV	intracerebroventrical
IPSC	inhibitory postsynaptic currents
LDCV	large dense core vesicle
LDTg	laterodorsal tegmental area
LEAP2	liver-expressed antimicrobial peptide 2
LGV	large granular vesicle
LH	lateral hippocampus
LTP	long term potentiation
MAPK	mitogen-activated protein kinase
MCR	melanocortin receptor
mRNA	messenger ribonulceic acid
MSD	mean square displacement
mTOR	mammalian target of rapamycin
Nacc	nucleus accumbens
NOS	nitric oxide synthase
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
OT	oxytocin
PFC	prefrontal cortex
PI-PLC	phosphoinositide-specific phospholipase C
PI3K	phosphatidylinositol-4,5-biphosphate 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
POMC	proopiomelanocortin
PPY	peptide YY
PRL	prolactin
PVN	paraventricular nucleus
QD	quantum dots
RNA	ribonucleic acid
ROS	reactive oxygen species
SEM	standard error of the mean
SEP	Solubility Enhancing Peptide
SON	supraoptic nucleus
SP-A	substance P inverse agonist of the ghrelin receptor
SSRI	selective serotonin reuptake inhibitor
SSV	small clear synaptic vesicle
TRH	Thyrotropin-releasing hormone
UCP2	uncoupling protein 2

VBS	visible burrow system
VIP	vasoactive intestinal peptide
VMH	ventromedial hypothalamus
VP	vasopressin
VTA	ventrotegmental area

Chapter 1 – Introduction

1 Neuropeptides

1.1 Neuropeptide definition and general function

Neuropeptides and peptide hormones may function as neuromodulators. Neuromodulators were discovered through the observation that the control of intracellular concentrations of cAMP, and activation of protein kinase C (PKC), regulates certain ionic currents, such as voltage sensitive calcium currents (DeRiemer et al., 1985; Siegelbaum et al., 1982), and the modulatory effect that several molecules, such as serotonin or luteinizing-hormone releasing factor, have on voltage-sensitive currents (Adams and Brown, 1980; Dudel, 1965; Dunlap and Fischbach, 1981). These molecules became known as neurotransmitters and neuromodulators. There is no boundary that clearly distinguishes the two. Both types of molecules transmit information between and within neural circuits. Vladimir Brezina suggests they can be distinguished by the spatial and temporal scale in which they act (Brezina, 2010). If a molecule acts locally and communicates information on a short-term scale (2-5 ms in the case of classical neurotransmitters (Salio et al., 2006)), as happens between the presynaptic terminal of one neurons and the post-synaptic terminal of another (the so called “wiring transmission” (Zoli and Agnati, 1996)), it may be called a neurotransmitter. If a molecule acts on multiple parts of the circuit and over a greater period of time than the timescale of synaptic activity (100-500 ms (Salio et al., 2006)), it can be called a neuromodulator. This type of communication is also referred to as “volume transmission”, since it happens over a bigger volume of extracellular fluid (Zoli and Agnati, 1996). Neuromodulators can transverse a long distance until they are recognized by their receptors (Ludwig and Leng, 2006). Biochemically, they can be subdivided in biogenic amines, namely dopamine, norepinephrine, serotonin and histamine, in purines, such as adenosine and adenine, in neuropeptides and in other classes of molecules such as nitric oxide (Brezina, 2010). There are cases when the same molecule can act as both a neurotransmitter and a neuromodulator, as in the case of acetylcholine (Picciotto et al., 2012). Finally, neuropeptides in particular, define one of the types of “neurotransmitter-mediated” communication between neurons, as detailed by Thomas Südhof (Südhof, 2008). The differences between classical neurotransmitters and neuropeptides are summarized in **Figure 1.1**. Burbach also defined neuropeptides as *“small proteinaceous substances produced and released by neurons through the regulated secretory route and acting on neural substrates”* (Burbach, 2011). Neuropeptides should also be distinguished from peptide hormones: the former are released and act within the nervous system, while the latter are transported through systemic circulation. Many neuropeptides are also peptide hormones, as in the case of oxytocin, vasopressin and ghrelin. In case of the latter, while it is a subject of debate if the levels of ghrelin produced in neurons at physiological relevant levels (Cabral et al., 2017), its role as a neuroactive peptide hormone is clear.

1.2 Neuropeptides - production and release

Neuropeptides are bioactive peptides, composed of 3-100 amino-acid residues (Salio et al., 2006), synthesized in neurons and released in a regulated manner. They modulate neural activity by activating molecular receptors (Burbach, 2010). These peptides and their receptors are coded by an immense variety of genes, widely subject to evolutionary processes (Niall, 1982), and further diversified by alternative splicing and post-translational modification (Perone et al., 1997). The classical neuropeptide gene families are: opioid, vasopressin/oxytocin, cholecystokinin/gastrin, somatostatin, F and Y-amide, calcitonin, natriuretic factor, bombesin-like peptide, endothelin, glucagon/secretin, corticotrophin-releasing factor-related, kinin and tensin, motilin, galanin, gonatropin-releasing hormone, neuropeptide B/W and insulin/relaxin (Burbach, 2011). Several neuropeptides do not belong to a specific gene family, such as the agouti-related peptide or prolactin (Burbach, 2011). **Table 1.1** shows several examples of neuropeptides and bioactive peptides released by neurons, grouped by local of release and function according to (Mains and Eipper, 2012).

In terms of evolutionary history, neuropeptides are thought to precede the development of fast-acting neurotransmitters since they can be found in cnidarians, like jelly fishes and the *Hydra,* while many canonical neurotransmitters like acetylcholine and the catecholamines are absent (Grimmelikhuijzen et al., 1999). This animal phylum is believed to be the most closely related to the first animals that developed a nervous system (Grimmelikhuijzen et al., 1999). The observed diversity of biologically active peptides is the result of the evolution of gene families from common ancestors, such as in the case of the gene for precursors of vasopressin and oxytocin, and the precursors to beta-endorphin (POMC), dynorphin and enkephalin (Niall, 1982). It is also the result of precursors that are processed into several bioactive peptides with different functions, such as is the case of POMC. Alternative splicing of the mRNAs of preprohormones, such as in the case of the preprotachykinin precursor that produces substance P, substance K and neurokinin A (Helke et al., 1990), also contributes increasing the diversity of neuropeptides. It was observed that bioactive peptide RNAs are also the target of post-translational gene editing. The bombesin-like peptide phyllolitorin found in the frog skin is gene edited, which changes a single aminoacid and produces two peptides that are more abundant in distinct tissues (Nagalla et al., 1994).

Neuropeptides are synthesized in the rough endoplasmic reticulum of the soma of neurons and stored exclusively in large granular vesicles (LGVs), also known as large dense core vesicles (LDCVs). These vesicles are transported to the neurites. During transportation, the synthesized neuropeptide precursors undergo cleavage and other types of processing (Mains and Eipper, 2012). Neurons often synthesize and co-store more than one neuropeptide in the same LGVs (Harling et al., 1991). These vesicles can also contain conventional neurotransmitters, as in the case of substance P-producing serotonergic neurons of the raphe nuclei and dorsal horn of the spinal cord (Pelletier et al., 1981). The neuropeptide-expressing neuron populations also belong to the canonical neurotransmitter-expressing populations.

Neuropeptide LGVs appear in lower number than neurotransmitter-carrying small clear synaptic vesicles (SSVs) and are stored in clusters in axons away from the presynaptic membrane (Salio et al., 2006; Shakiryanova et al., 2005) and almost every other part of the neuron (Morris and Pow, 1991). Like SSVs, the exocytosis of LGVs is dependent on the elevation of the intracellular Ca^{2+} concentration triggered by the activation of voltage-gated Ca^{2+} channels (Shakiryanova et al., 2005), and is sensed by the same SNARE proteins (Martin, 1994). In contrast with SSVs, LGVs release happens upon lower overall intracellular concentrations of Ca^{2+} (Ghijsen and Leenders, 2005) and over longer periods of time, after repetitive neuron firing. These two facts are explained by the necessity of continuous trains of action potentials in order for the intracellular concentration of Ca^{2+} to increase high enough away from the presynaptic terminals (Ghijsen and Leenders, 2005). LGV release also depends on the pattern of activity of the neuron. For instance, in hypothalamic oxytocin neurons the release of oxytocin from dendrites can be primed by agents that mobilize intracellular Ca^{2+} without affecting electrical activity, making the pool of releasable oxytocin sensitive to spikes (Ludwig et al., 2002). Neuropeptides can also be released quickly or slowly depending on the frequency of bursting activity, as demonstrated by Shakiryanova and colleagues in an *in vivo* model (Shakiryanova et al., 2005). Once released into the extracellular space, neuropeptides have relatively long half-lives, as in the case of the ~20 min half-life of oxytocin and vasopressin, and are eventually degraded by several peptidases (Roques et al., 2012). Overall, a variety of neuropeptides allows the communication of specific modulator information and their release dynamics allows the communication of temporal information (Hökfelt et al., 2000).

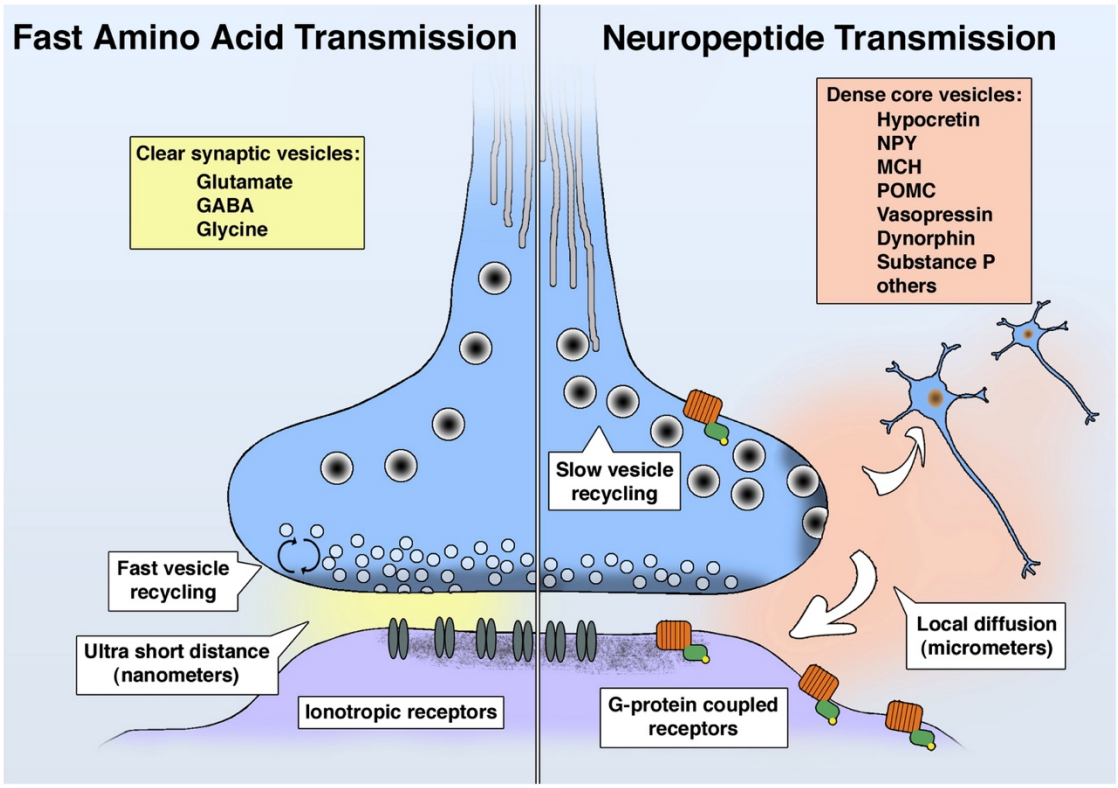


Figure 1.1 – Comparison between fast amino acid neurotransmission and neuropeptide transmission. Clear synaptic vesicles carry canonical neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA) and glycine, that are released on presynaptic terminals. These vesicles are quickly recycled and the released neurotransmitters travel the short distance between the presynaptic

and postsynaptic terminal. Neurotransmitters can activate ionotropic receptors on the postsynaptic terminal. Dense core vesicles store neuropeptides such as hypocretin, neuropeptide Y, melanin-concentrating hormone, pro-opiomelanocorticotropin, vasopressin, dynorphin, substance P among others. Upon release, these neuropeptides can travel longer distances and will activate G-protein coupled receptors on postsynaptic cells and more distant cells. Adapted from (van den Pol, 2012)

Table 1.1 - Examples of neuropeptides and bioactive peptides found in the brain, organized by type and function. * indicates peptides that will be explored in detail in the introduction. Based on (Mains and Eipper, 2012)

Type	*Peptide*	*Endocrine function/Most well-known function*	*Examples of other functions*	*References*
Hypothalamic releasing factors *– peptides that act on the pituitary gland and stimulate the release of other peptides*	**Corticotropin releasing hormone (CRH)**	The major regulator of the secretion of ACTH and other POMC-derived peptides	Coordinates the autonomic, immunological, and behavioral response to stress. Is connected to affective and anxiety disorders	(Owens and Nemeroff, 1991)
	Growth hormone releasing hormone (GHRH)	Stimulates the release of growth hormone. Stimulates the growth of growth hormone releasing cells	Regulates sleep-wake cycle, modulates feeding behavior and is implicated in obesity	(Billestrup et al., 1986 ; Cai and Hyde, 1999; Ehlers et al., 1986)
	Gonadotropin releasing hormone (GnRH)	Stimulates the synthesis of luteinizing hormone and follicle-stimulating hormone	Regulates reproduction. The genetic absence of GnRH results in hypogonadism, infertility and failure of sexual maturation	(Wu et al., 2010)
	Somatostatin	Inhibits the release of thyrotropin-releasing hormone and growth hormone on the hypothalamus	Involved in learning, cognition and locomotion. Cortistatin, a somatostatin-related peptide, depresses neural activity and induces slow-wave sleep	(de Lecea et al., 1996)
	Thyrotropin-releasing hormone (TRH)	Stimulates the release of thyroid-stimulating hormone, prolactin and growth hormone	Its receptors are involved in sleep, learning, memory, anxiety and depression	(Zeng et al., 2007)
Pituitary hormones *– peptides that are released from pituitary gland into circulation and produce effects on distal organs. They can also produce effects in the central nervous system*	**Adrenocorticotropic hormone (ACTH)**	Derived from POMC. Stimulates the release of glucocorticoids from the adrenal cortex	Apart from the adrenal effects, ACTH stimulates lipolysis and modulates the immune system. Absence of the MC2R receptor of ACTH leads to hypoglycemia and susceptibility to infections, among other effects	(Metherell et al., 2005)
	α-melanocyte stimulating hormone (α-MSH)	Derived from POMC. Stimulates production of melanin by skin melanocytes	Since it is an equally high affinity ligand for all the melanocortin receptors (MCR), the effect depends on the stimulated receptor: changes in pigmentation (MC1R), feeding behavior and	(Cone, 2006)

			natriuresis (MC3R), feeding behavior and erectile function (MC4R)	
	β-endorphin	Derived from POMC. Has 18 to 33 times more potent analgesic activity than morphine. Best endogenous ligand of μ-opioid receptor (MOR)	In the central nervous system, it regulates feeding, sexual behavior and learning. In the periphery it is a regulator of cardiovascular and immune system function	(Akil et al., 1984; Loh et al., 1976)
	Growth hormone (GH)	Stimulates somatic growth (in a manner highly connected to insulin-like growth factor)	Stimulates protein metabolism and regulates the utilization of blood glucose and aminoacids. It also regulates sexual maturation and the production of sexual steroids	(Kopchick and Andry, 2000)
	Prolactin (PRL)	Stimulates milk production on female mammary glands	Prolactin receptors are widely expressed throughout the body and regulate salt and water balance and levels of sexual hormones. Also regulate cell growth and differentiation in blood cell formation, angiogenesis and the immune system	(Bole-Feysot et al., 1998; Mancini et al., 2008)
Peptides found on the brain and the gastrointestinal tract	**Neurotensin**	It was isolated for its peripheric hypotensive action. In the gastrointestinal tract it regulates pancreatic secretion, colonic motility and gastric secretion	In the central nervous system, produces hypothermia, stimulates the release of several pituitary peptides including ACTH and GnRH, reduces food intake and is potent analgesic	(Billestrup et al., 1986; Cador et al., 1986; Clineschmidt and McGuffin, 1977; Fuxe et al., 1984; Rowe et al., 1995)
	Motilin	Its gene has high homology to the ghrelin gene. Found at the highest concentrations in the gastroduodenal region. Stimulates the phase 3 of the migrating motor complexes in the stomach and duodenum	In the central nervous system, induces the release of growth hormone and increases food intake. Reduces anxiety	(Folwaczny et al., 2001; Momose et al., 1998)
	Substance P	Part of the tachykinin family. Found in the majority of the peripheral and central nervous system. Also released in the enteric nervous system. Promotes motility in the gastrointestinal tract and regulates emesis. Regulates nociception.	Regulates vasodilation and inflammation. It is released from the spinal cord upon various nociceptive stimuli. Also promotes learning and is associated with stress and anxiety	(Honoo et al., 2013; Hoppe et al., 2018; Huston and Hasenöhrl, 1995; Niel, 1991)

	Cholecystokinin (CCK)	Released from cells of the small intestine. Induces contraction of the gallbladder, secretion of pancreatic enzymes and inhibits gastric motility	In the central nervous system increases anxiety and nociception, decreases food intake, and regulates memory and learning	(Dockray, 2012; Herranz, 2003)
	Ghrelin*	Secreted from the stomach and proximal duodenum. Strongly stimulates the secretion of growth hormone	In the central nervous system it is involved in many functions such as regulation of food intake, learning and memory, sleep, anxiety and depression	(Kaiya et al., 2011)
Opiate peptides	**Dynorphin**	A very potent opioid peptide. Best endogenous ligand for κ-opioid receptor (KOR)	Activation of KOR produces analgesia. It also regulates learning and memory, emotional responses and stress	(Schwarzer, 2009)
	Enkephalin	Best endogenous ligand for δ-opioid receptor (DOR)	Activation of these receptors produces the well-described effects of morphine including analgesia, euphoria, decreased respiratory rate and sedation.	(Al-Hasani and Bruchas, 2011)
	β-endorphin	As described above	As described above	
Neurohypophyseal peptides *– peptides that are released from the hypothalamus into the brain and into systemic circulation*	**Oxytocin***	Produced by magnocellular secretory neurons of the hypothalamus. Induces milk production by stimulation of the secretion of prolactin. Involved in parturition.	Projection neurons from the hypothalamus release oxytocin in several brain regions. It is involved in social recognition and bonding, sexual behavior, paternal and maternal behavior, memory, anxiety and depression	(Lee et al., 2009)
	Vasopressin*	Produced by magnocellular secretory neurons of the hypothalamus. Has antidiuretic action and increases blood pressure. Regulates fatty acid metabolism and control release of pancreatic hormones	Projection neurons from the hypothalamus release vasopressin in several brain regions. It regulates social behavior, learning and memory, water and food intake and is implicated in depression and anxiety. Regulates the secretion of ACTH and CRH	(Caldwell et al., 2008)
Peptides found on brain and endocrine system	**Calcitonin gene-related peptide (CGRP)**	Found in sensory nerve terminals. Co-localizes with substance P. Production and secretion is increased during inflammation. It is a highly potent	Its antagonism alleviates migraine. Believed to be involved in arthritis, cardiovascular disease, obesity and diabetes	(Russell et al., 2014)

		vasodilator and involved in pain pathways and wound healing		
	Vasoactive intestinal peptide	Widely distributed in the central and peripheral nervous system. Mainly acts as a vasodilator and regulator of GI tract motility	Finely tunes other neuromodulator systems. Is involved in the sleep-wake cycle, learning and memory. Regulates the release of pituitary hormones	(Chaudhury et al., 2008; Rosténe, 1984)
Neuropeptides *– peptides found almost exclusively on the brain*	**Neuropeptide Y (NPY)***	A member of the NPY family. Widely distributed in the central nervous system and sympathetic system. Increases food intake in the hypothalamus	Is a potent vasoconstrictor. Regulates sleep-wake cycles, stress and memory. In implicated in anxiety, depression and seizures	(Pedrazzini et al., 2003; Wu et al., 2011)
	Peptide YY (PYY)	A member of the NPY family. Expressed in the GI tract. Inhibits stomach emptying and the secretion of stomach acid and pancreatic enzymes	In the periphery reduces food intake. If administered to the brain has a similar food intake stimulating effect as NPY	(McGowan and Bloom, 2004)
	Galanin	Its receptor is widely distributed through the peripheric organs and the central nervous system	Involved in the regulation of sleep, energy expenditure, reproduction and cognition	(Lang et al., 2007)
	Neuromedin-U	A member of the neuromedin family of peptides. Widely distributed through peripheric organs and the central nervous system. Regulates GI tract motility and blood pressure	In the central nervous system, regulates nociception and stress response, reduces food intake and controls circadian rhythm	(Martinez and O'Driscoll, 2015)

2 Neuropeptide Receptors

Neuropeptides convey their information via the activation or inhibition of membrane receptors. Almost all of these receptors are seven-transmembrane domain G-protein coupled receptors (**Figure 1.2**). Two exceptions are the ionotropic receptor of FMRFamide found on the snail *Helix aspersa* and the neurotensin receptor sortilin, a large protein with a single transmembrane sequence (Hökfelt et al., 2003). The first neuropeptide GPCR to be cloned was the substance P receptor neurokinin 2 receptor (Masu et al., 1987). Of the 550 GPCR genes present in the human genome, neuropeptides are known ligands for about 20% of them (Hökfelt et al., 2003) and of the mammalian 67 families of GPCRs, 35 are neuropeptide subfamilies. These amount to 80 individual GPCRs (Gainer, 2016). These GPCRs mainly belong to the rhodopsin family, according to the GRAFS classification (Schioth and Fredriksson, 2005). **Table 1.2** enumerates the known families of neuropeptide receptors and gives an overview of the effects of the agonism of these receptors.

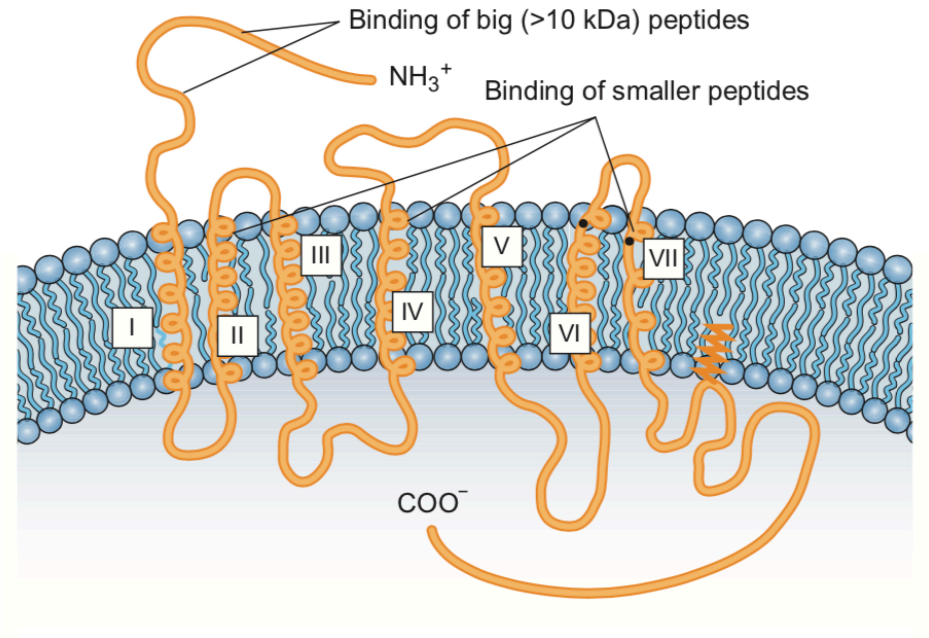


Figure 1.2 – Illustration of the general structure of 7 transmembrane domain G-protein coupled receptors that bind neuropeptides. The extracellular domain tends to bind bigger peptides while smaller peptides tend to bind within the transmembrane domain and in the extracellular loops. Adapted from (Mains and Eipper, 2012).

In terms of expression, the receptors for neuropeptides are mainly, but not exclusively, located away from the synapse and from the sites of release of their ligands (Merighi et al., 2011). They can be expressed in the membrane surface of any part of the neuron, as showed by the expression pattern of substance P receptor neurokinin 1 receptor (Mantyh, 2002) or NPY receptor Y_5 (Acuna-Goycolea et al.,

2005). Neuropeptide receptors have a much higher binding affinity than that of classical neurotransmitter receptors, which is consistent with their involvement in “volume transmission”, the low concentration at which they are stored in LDCVs and the dependence on long distance diffusion (Merighi et al., 2011). The expression profile of neuropeptide and neuropeptide GPCR genes in the brain varies widely (Burbach, 2011), but certain regions express an exceptional variety of these genes. This is the case of the paraventricular and supraoptic nucleus of the hypothalamus, which expresses neuropeptide GPCRs from 33 of the known 35 subfamilies (Hazell et al., 2012).

Activation of neuropeptide GPCRs produces conformational changes that catalyze the exchange of GDP for GTP on Gα subunit of the coupled G proteins and lead to the dissociation of Gα-GTP and Gβγ. The activated intracellular pathway depends mainly on the Gα subunit type coupled to the receptor: $G\alpha_s$ activate adenylyl cyclases and stimulate the production of cAMP; $G\alpha_{q/11}$ activates phospholipase C, leads to increases in intracellular Ca^{2+} and activates the pathway of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK); $G\alpha_{i/o}$ activates several phospholipases and phosphodiesterases and inhibits adenylyl cyclases (Gainer, 2016). Gβγ can also activate adenylyl cyclases, phospholipases, MAP kinases and ion channels. β-arrestins, cytosolic adaptor proteins of GPCRs, also participate in the signaling of neuropeptide receptors (Jean-Charles et al., 2017). The majority of neuropeptide GPCRs produce signaling through $G\alpha_{q/11}$ (Hazell et al., 2012). The same neuropeptide GPCR can be coupled to different G protein subunits. For instance, vasopressin receptor V1a couples to both $G\alpha_{i/o}$ and $G\alpha_{q/11}$ depending on the phase of the cell cycle of the cell that expresses it (Abel et al., 2000). Another example is the substance P and neurokinin receptor NK1, which exists in two high-affinity ligand binding states: the one with the highest affinity couples to $G\alpha_s$ and the one with the lowest affinity couples to $G\alpha_{q/11}$ (Holst et al., 2001). GPCRs for different neuropeptides elicit intracellular signaling pathways that interact with each other. An example of this interaction is the CRF_1 receptor-mediated release of ACTH in the hypothalamus, through activation of adenylyl cyclase. This process is amplified by the activation of protein kinase C by agonism of vasopressin V1b receptors found in the same cells (Labrie et al., 1984).

The effects of the activation of neuropeptide receptors in neurons can be grouped in effects on the presynaptic and postsynaptic terminals (Nadim and Bucher, 2014), and effects on the excitability and spiking of neurons. Neuropeptides are known to modify the release probability of GABA and glutamate from presynaptic terminals. For example, by reducing Ca^{2+} influx into the presynaptic terminals of Schaffer collaterals, NPY inhibits glutamatergic transmission in the hippocampus (Colmers et al., 1988). Another example is the orexin-mediated enhancement of presynaptic glutamate and GABA release on hypothalamic neurons (Van Den Pol et al., 1998). On the post-synaptic region, neuropeptides can modify synaptic strength. Two examples are the enhancement of glutamatergic transmission in the CA1 by pituitary adenylate cyclase activating polypeptide 38 (PACAP38) (Roberto et al., 2001), most likely through phosphorylation of AMPA receptors (Toda and Huganir, 2015), and a similar effect induced by the activation of the ghrelin receptor GHSR1a in hippocampal neurons (Ribeiro et al., 2014). Finally, neuropeptides are well known to modify the excitation state of neurons. This is the case of the

hyperpolarization and consequent reduction of firing that occurs in thalamic neurons through the activation of the NPY1R (Sun et al., 2001). This is achieved by NPY1R-mediated activation of G-protein-activated inwardly rectifying K^+ channels (GIRK), which in many tissues are indirectly activated by several neuromodulators, such as dopamine, adenosine and the neuropeptide somatostatin (Lüscher and Slesinger, 2010). By signaling through GPCRs, neuropeptides induce changes in cellular physiology over a more prolonged timeframe than classical neurotransmitters that activate ion channel receptors (Gainer, 2016).

Table 1.2 - Neuropeptide GPCR families and examples of their ligands and functions of their activation. Based on (Gainer, 2016). For a brief description of the functions of already mentioned peptides refer to table 1.1

Name	*Class*	*Well-known Neuropeptide Ligand*	*Overview of the effects of agonism of the receptor*	*Reference*
Angiotensin	A (rhodopsin-like)	Angiotensin	Angiotensin 2 receptor is widely expressed in the brain. The knockout of its gene impairs memory and learning and reduces exploratory behavior	(Gallo-Payet et al., 2011; Maul et al., 2008)
Apelin	A (rhodopsin-like)	Apelin	Expressed in the hypothalamus. Reduces vasopressin secretion and is involved in the central control of fluid homeostasis	(Reaux et al., 2001)
Bombesin	A (rhodopsin-like)	Neuromedin B	Controls satiety, circadian rhythms and other behaviors. Potent effects on the gastrointestinal, respiratory and immune system	(Ramos-Alvarez et al., 2015)
Bradykinin	A (rhodopsin-like)	Bradykinin	Potent systemic vasodilator. B2 is present in primary sensory neurons and is involved in initial nociceptive response	(Lopes et al., 1993)
Calcitonin	B (secretin receptor family)	Calcitonin, Amylin, CGRP	Suppresses osteoclast actitivity. In the central nervous system, suppresses food and water consumption. Also, CGRP effects as mentioned above	(Wimalawansa, 1997)
Cholecystokinin	A (rhodopsin-like)	Cholecystokinin (CCK)	Widely expressed in the central nervous system. CCK is released by a specific population of interneurons and its receptors are found in pyramidal neurons. Involved in modulation of neural excitability and synaptic transmission, implicated in anxiety and epilepsy	(Lee and Soltesz, 2011)

Corticotropin-releasing factor	B (secretin receptor family)	CRH	Refer to table 1.1	
Endothelin	A (rhodopsin-like)	Endothelin-1 (ET-1)	Causes vasoconstriction and increases blood pressure. In the central nervous system, ET-1 regulates blood pressure and alters nerve conduction in the peripheral nervous system	(Dashwood and Loesch, 2010)
Galanin	A (rhodopsin-like)	Galanin	Refer to table 1.1	
Ghrelin*	A (rhodopsin-like)	Ghrelin	Refer to table 1.1	
Glucagon	B (secretin receptor family)	Glucagon-like peptide-1	Potent inducer of the secretion of insulin. In the brain, regulates feeding, body temperature and blood pressure	(Blazquez et al., 1998)
Gonadotropin-releasing hormone	A (rhodopsin-like)		Refer to table 1.1	
Kisspeptin	A (rhodopsin-like)	Kisspeptin	In the hypothalamus regulates GnRH secretion, reproductive function and puberty onset. Decreases food intake in fasting animals	(Orlando et al., 2018)
Melanin-concentrating hormone	A (rhodopsin-like)	Melanin-concentrating hormone (MCH)	Potently increases food intake. Also involved in arousal and neuroendocrine control	(Macneil, 2013)
Melanocortin	A (rhodopsin-like)	ACTH, α-MSH	Refer to table 1.1	
Motilin	A (rhodopsin-like)	Motilin	Refer to table 1.1	
Neuromedin U	A (rhodopsin-like)	Neuromedin	Refer to table 1.1	
Neuropeptide FF/AF	A (rhodopsin-like)	Neuropeptide FF	Involved in pain modulation, control of blood pressure, feeding behavior and body temperature	(Mankus and McCurdy, 2012)
Neuropeptide S	A (rhodopsin-like)	Neuropeptide S	Potently regulates wakefulness and reduces anxiety	(Xu et al., 2004)

Neuropeptide W/B	A (rhodopsin-like)	Neuropeptide W	Expressed in the periphery and hypothalamus. Suppresses food intake and increases body temperature	(Takenoya et al., 2012)
Neuropeptide Y*	A (rhodopsin-like)	NPY, PYY	Refer to table 1.1	
Neurotensin	A (rhodopsin-like)	Neurotensin	Refer to table 1.1	
Opioid	A (rhodopsin-like)	β-endorphin, dynorphin, enkephalin	Refer to table 1.1	
Orexin	A (rhodopsin-like)	Orexin	Increases food intake. Involved in the maintenance of wakefulness, and implicated in sleep disorders, obesity, stress and addiction	(Tsujino and Sakurai, 2009)
Parathyroid hormone	B (secretin receptor family)	Tuberoinfundibular peptide of 39 residues (TIP39)	Expressed in the hypothalamus. Parathyroid hormone receptor 2 modulates stress responses and nociception, and induces prolactin release. Implicated in anxiety	(Dobolyi et al., 2012)
Peptide P518	A (rhodopsin-like)	P518, also known as QRFP or 26RFamide	Increases food intake and locomotor activity	(do Rego et al., 2006)
Prokineticin	A (rhodopsin-like)	Prokinectin 2 (PK2)	PK2 potently inhibits food intake in the hypothalamus	(Gardiner et al., 2010)
Prolactin-releasing peptide	A (rhodopsin-like)	Prolactin-releasing peptide	Induces prolactin secretion in the hypophysis. Also inhibits food intake, regulates stress and stimulates the release of somatostatin, GnRH and oxytocin	(Takayanagi and Onaka, 2010)
Relaxin	A (rhodopsin-like)	Relaxin-3	Increases food intake and regulates reproduction	(McGowan et al., 2009)
Somatostatin	A (rhodopsin-like)	Somatostatin	Refer to table 1.1	
Tachykinin	A (rhodopsin-like)	Substance P and Neurokinins	Refer to table 1.1	

Thyrotropin-releasing hormone	A (rhodopsin-like)	TRH	Refer to table 1.1	
Urotensin	A (rhodopsin-like)	Urotensin II (in the case of central nervous system)	Stimulates the release of prolactin, TRH and ACTH. Involved in mood and sleep regulation and the central control of cardiovascular function. Activation of the receptor increases locomotion	(Lihrmann et al., 2013)
Vasopressin/oxytocin*	A (rhodopsin-like)	Vasopressin and Oxytocin	Refer to table 1.1	
VIP/PACAP	B (secretin receptor family)	VIP and Pituitary adenylate cyclase-activating peptide (PACAP)	Refer to table 1.1	

3 Functions of Neuropeptides

Neuropeptides modulate almost all physiological systems. Tables 1.1 and 1.2 shows many examples of neuropeptides that regulate reproduction, sexual behavior, lactation, stress responses, sleep wake cycles, feeding behavior and social behavior. Neuropeptides are also important regulators in the gastrointestinal tract and cardiovascular system.

In this work we explored the role of neuropeptides in feeding behavior, with an emphasis on ghrelin, and in social behavior, with an emphasis of neuropeptide Y.

3.1 Neuropeptides and feeding behavior

Feeding is controlled by the brain by sensing the organisms metabolic state and activating homeostatic mechanisms to maintain a favorable balance between acquisition and utilization of nutrients. Information about the nutritional state is communicated to the brain through peptide hormones, such as leptin and ghrelin, which activate neuropeptide-producing neurons in the hypothalamus. In particular, the arcuate nucleus (Arc) of the hypothalamus works as a sensor for peripheric hormones signaling metabolic state (Barsh and Schwartz, 2002).

Activation of NPY-producing neurons in the Arc increases feeding. These neurons release NPY locally and into enervated regions, namely the paraventricular nucleus (PVN) and perifornical area/lateral hypothalamus, (PF/LH). In these regions, NPY receptors Y1R, Y5R and Y2R are activated. NPY neurons are of high importance for feeding since their ablation, by recourse to targeted expression of the diphtheria toxin receptor, produces an acute and sustained reduction on feeding (Gropp et al., 2005). Their chemogenetic activation produces an also acute increase on food intake (Krashes et al., 2011). These neurons also produce agouti-related peptide (AgRP), an orexigenic peptide. A number of molecules and neuropeptide stimulate and inhibits these neurons. For example, they are inhibited by circulating leptin and insulin, and excited by corticosterone and ghrelin (Leibowitz and Wortley (2009)). NPY by itself is an extremely potent inducer of feeding, as evidenced by the increased feeding in rats that received NPY injection directly into the paraventricular nucleus (Stanley and Leibowitz, 1985). The expression of AgRP by these neurons increases with fasting (Hahn et al., 1998). It is noteworthy that the knockout for NPY does not show any relevant phenotype in respect to food-intake, body weight and energy balance (Bannon et al., 2000b). This is also the case for the knockout of AgRP and double knockout of AgRP/NPY (Qian et al., 2002). This may be reconciled by the fact that both knockouts for AgRP and NPY can show a reduction in hyperphagia following fasting and, in the case of AgRP KO, decreased body weight in old age (Wortley et al., 2005). These neurons also release the GABA neurotransmitter, and may produce still undefined neuropeptides important for their role in feeding (Flier, 2006).

The Arc also contains a distinct neuron population that reduces feeding when activated. These neurons are characterized by the expression of neuropeptide precursors pro-opiomelanocortin (POMC), and neuropeptide cocaine and amphetamine responsive transcript (CART). POMC is cleaved into melanocortins, such as the α-melanocyte-stimulating hormone (α-MSH), which suppresses feeding by activating the melanocortin receptors (Schwartz et al., 2000). Both the mouse knockout for POMC and for melanocortin-4 receptor (MC4R), a widely expressed melanocortin receptor in the brain (Mountjoy et al., 1994), are found to be obese and hyperphagic (Challis et al., 2004; Huszar et al., 1997). AgRP is an endogenous antagonist of the MC4R. In contrast with the NPY/AgRP neurons, POMC neurons are stimulated by the activation of the leptin and insulin receptors. The plasmatic levels of leptin and insulin are proportional to the amount of adipose tissue in the organism, thus signaling to the hypothalamus an overabundance of stored energy (Benoit et al., 2004).

These Arc neurons regulate metabolic homeostasis by conveying information to other brain regions. For instance, the Arc neurons project to the paraventricular (PVN) neurons of the hypothalamus and their release of AgRP or α-MSH controls the release of thyrotropin-releasing hormone (Fekete et al., 2000; Legradi and Lechan, 1999). This peptide controls the activity of the thyroid system which regulates the overall energy expenditure of the organism. The PVN neurons also synthesize other neuropeptides that regulate feeding, including corticotropin-releasing hormone and oxytocin (Verbalis et al., 1995), which are anorexigenic, and galanin (Crawley, 1999), which is orexigenic. Urocortins are also anorexigenic neuropeptides expressed in the Arc and PVN (Reyes et al., 2001). They activate CRH receptors, and reduce food intake to varying degrees (Pan and Kastin, 2008). The Arc also synapses with neurons of the lateral nucleus and the adjacent perifornical area of the hypothalamus, which express melanin concentrating hormone (MCH), a plasmatic peptide hormone that increases feeding (Shimada et al., 1998). The perifornical area also produces the orexigenic neuropeptides hypocretin/orexin 1/A and 2/B (Sakurai et al., 1998).

4 Ghrelin – an important peptide for feeding

4.1 The ghrelin peptide

Ghrelin is a peptide hormone that increases food intake by activating NPY neurons in the Arc (Shintani et al., 2001). It was identified for being the endogenous ligand to the growth hormone secretagogue receptor type 1 (Kojima et al., 1999). It is part of the ghrelin-motilin peptide family and both ghrelin/motilin precursors and their corresponding receptors share around 50 % amino acid sequence homology (Tomasetto et al., 2000). Ghrelin human gene is found on the short arm of the chromosome 3 (3p25-26). This gene contains 6 exons and 4 introns. The secreted peptide contains 28 amino-acids and is coded by exons 1 and 2. The transcript produces the precursor prepro-ghrelin of 117 aa (Korbonits et al., 2004). This precursor is cleaved into its N-terminal and the 94 aa pro-ghrelin (Garg, 2007) and the latter is cleaved by prohormone convertase 1/3 into ghrelin 1-28, and carboxypeptidase-like B enzyme into ghrelin 1-27 (Hosoda et al., 2003). The active form of ghrelin requires its acylation

with an octanoyl group on serine-3, which is performed by the enzyme ghrelin O-acyltransferase (GOAT) in the endoplasmic reticulum (Yang et al., 2008).

Ghrelin is mainly secreted to the plasma by X/A-like cells located in the stomach and proximal intestine, in both humans and rats (Date et al., 2000), and its plasmatic levels peak immediately before each meal (Cummings et al., 2001). Throughout the day, the plasmatic levels of ghrelin oscillates, with peaks before meals and throughs after food ingestion (Cummings et al., 2001). Ghrelin is also produced in the jejunum, ileum, colon, lungs (Date et al., 2000; Hosoda et al., 2000; Sakata et al., 2002), gonads, pancreatic islets and placenta (Dezaki and Yada, 2012; Gaytan et al., 2003; Gualillo et al., 2001; Hattori et al., 2001; Mori et al., 2000; Tena-Sempere et al., 2002; Volante et al., 2002). Ghrelin gene expression has been detected in the hypothalamic arcuate nucleus (Lu et al., 2002), in axons that enervate the lateral hypothalamus and ventromedial, dorsomedial and paraventricular nuclei of the hypothalamus (Cowley et al., 2003a), and in the ependymal layer of the third ventricle (Cowley et al., 2003b).

Ghrelin gene expression in the stomach is upregulated by glucagon (Wei et al., 2005), which is highest during food restriction, and is inhibited by leptin (Kamegai et al., 2004). Ghrelin expression is also regulated by glucose, insulin, melatonin, growth hormone, somatostatin and the parasympathetic nervous system (Korbonits et al., 2004). X/A-like cells produce numerous membrane surface receptors that enable the regulation of ghrelin secretion by these molecules (Engelstoft et al., 2013). The expression of the peptide is regulated by the feeding status of the organism. Levels of plasmatic ghrelin increase two-fold before each meal (Cummings et al., 2001) and decrease after the meal to an extent proportional to the caloric load consumed (Williams et al., 2003). Fasting increases ghrelin expression in the stomach (Torsello et al., 2003) and in mice a prolonged period with 60% reduction in food intake leads to a marked increase in plasmatic ghrelin levels (Lutter et al., 2008). Obese subjects have been characterized as displaying lower levels of plasmatic ghrelin when compared to lean individuals (Rosicka et al., 2003)

Ghrelin is capable of crossing the blood brain barrier (BBB) in both directions (Banks et al., 2002), and this process is linked to the metabolic status of the organism (Banks et al., 2008). Ghrelin crosses the BBB to different extents according to brain region. The olfactory bulb and occipital cortex take up the highest amounts, followed by the hypothalamus, the hippocampus and then the rest of the brain (Diano et al., 2006b).

The knockout for ghrelin shows normal growth and food intake and no gross alterations to behavior. However, these animals show lower body fat when given a high fat diet (Kojima and Kangawa, 2005). These animals also have lower spine density in the CA1 region of the hippocampus, perform worse than controls in the novel-object recognition test (Diano et al., 2006b) and show anxiety-like behavior after restraint stress (Spencer et al., 2012).

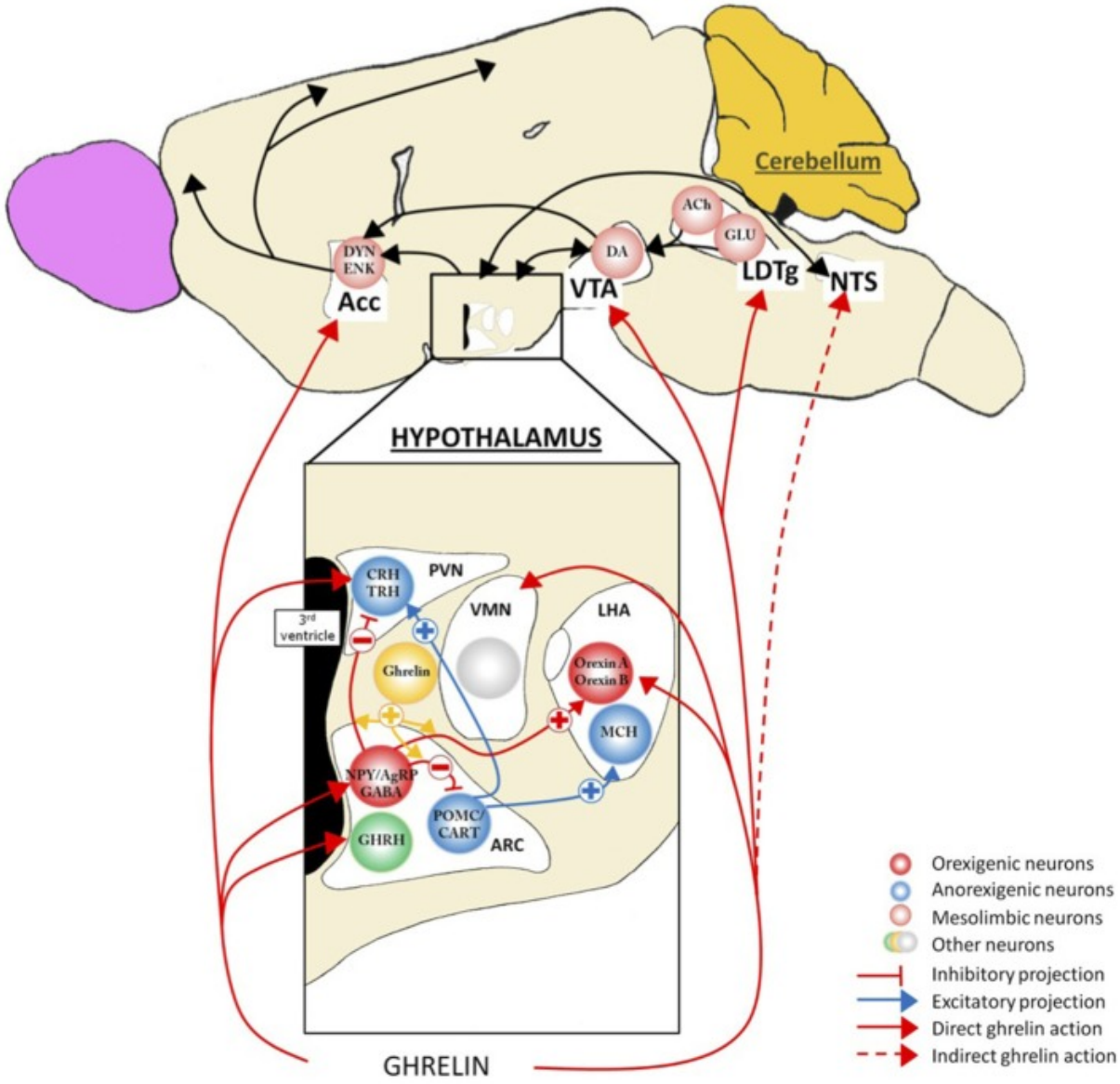


Figure 1.3 – Sites of action of ghrelin on the hypothalamus, meso-cortico-limbic pathway and brainstem. This figure illustrates the hypothalamic circuit that regulates food intake and the sites of action of ghrelin. PVN, paraventricular nucleus; CRH, corticotrophin-releasing hormone; Thyrotropin-releasing hormone; VMN, ventromedial nucleus; LHA, lateral hypothalamus; ARC, arcuate nucleus; MCH, melanin-concentrating hormone; DYN, dynorphin; ENK, enkephalin; DA, dopamine; Acc, Nucleus Accumbens; VTA, ventrotegmental area; LDTg, laterodorsal tegmental area; NTS, nucleus tractus solitarius; Ach, acetylcholine; GLU, glutamate; Adapted from (Mequinion et al., 2013)

4.2 The ghrelin receptor

Ghrelin increases feeding by increasing the activity of the NPY/AgRP neurons of the Arc (**Figure 1.3**). This is validated by the absence of this effect in ARC-ablated mice injected with ghrelin (Cabral et al., 2014). This effect is produced by the activation of the ghrelin receptor growth hormone secretagogue receptor 1a (GHSR1a) (Kojima et al., 1999). The gene for this receptor is located on chromosome 3 at 3q26.2, which encodes a full-length receptor GHSR1a, and a shortened version GHSR1b (McKee et al., 1997), which does not bind growth hormone secretagogue (Howard et al., 1996). GHSR1a is a family A GPCR, has 366 amino-acids and a size of around 41 kDa (Camina et al., 2004). The GHSR1a

is most closely related to the motilin receptor (52% homology), neurotensin recepetor 1 and 2 (33-35% homology) and neuromedin U receptor 1 and 2 (30% homology) (Tan et al., 1998). The ghrelin receptor has high sequence similarity across mammalian species, which is indicative of its evolutionary importance (Kaiya et al., 2014).

The ghrelin receptor heterodimerizes with other GPCRs, which greatly increases the complexity of its signaling. GHSR1a heterodimerizes with D1 dopamine receptor (D1R) and activation of the receptor by ghrelin enhances dopamine signaling through D1R (Jiang et al., 2006). These heterodimers were found in hippocampal neurons. It was found that in these complexes, D1R activation produced signal transduction via $G\alpha_q$-PLC-IP_3-Ca^{2+} rather than through the canonical $G\alpha_s$ pathways (Kern et al., 2015). Activation of the receptor in this manner enables the activation of CAMKII and phosphorylation of AMPA receptor at serine 831, molecular events important for hippocampal dependent memory formation. Antagonism of the ghrelin receptor prevents these effects from being produced by the agonism of D1R. These effects were also validate *in vivo* by the ability of the same ghrelin antagonism to block D1R agonism induced improvements in hippocampal dependent memory (Kern et al., 2015). GHSR1a/D2R heterodimers are also described in hypothalamic neurons (Kern et al., 2012). GHSR1a also heterodimerizes with the serotonin receptor 5-HT_{2C}, which reduces the calcium influx upon GHSR1a activation, an effect that is prevented by blocking of the serotonin receptor (Schellekens et al., 2013). GHSR1a also complexes with melanocortin receptor 3 (MC3R) which amplifies MC3R intracellular signaling in a manner dependent on GHSR1a activity (Rediger et al., 2011).

GHSR1a is expressed in the arcuate, paraventricular and ventromedial nucleus of the hypothalamus. It is also found on the cortex, ventral tegmental area, substantia nigra, pons, medulla oblongata, amygdala, olfactory bulb and hippocampus (Guan et al., 1997a; Mani et al., 2014; Zigman et al., 2006). In the hippocampus, the receptor transcript is detectable in all subregions (Zigman et al., 2006). In the CA1, GHSR1a can be found in the soma, dendrites (Berrout and Isokawa, 2012), and in hippocampal neuron cultures it can be found in excitatory synapses (Ribeiro et al., 2014). mRNA for the receptor becomes detectable after postnatal day 7 (P7) on rodents (Ribeiro et al., 2014). GHSR1a is also expressed in the lungs, heart, arteries, thyroid, liver, stomach, pancreas, small and large intestines, immune cells and adipose tissue (Gnanapavan et al., 2002; Guan et al., 1997b; Hattori et al., 2001; Kojima et al., 2001).

4.3 Intracellular signaling of the ghrelin receptor

This receptor belongs to the G protein-couple receptors, contains seven transmembrane helices and has an extracellular N-terminus and intracellular C-terminus (Conn, 2010). Both G_s and G_q signaling pathways can be activated, depending on the tissue where it is expressed (Soares et al., 2008). The intracellular signaling produced by the ghrelin receptor may be divided in signaling involving calcium, AMP activated protein kinase (AMPK), phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K), mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK). In the case of

the effects of ghrelin on the central nervous system, the most relevant types of signaling are connected to calcium, AMPK, PI3K and MAPK.

Activation of the ghrelin receptor on growth hormone releasing neurons of the pituitary gland increases intracellular levels of calcium, through its coupling to protein $G_{q/11}$ and activation of the phosphoinositide-specific phospholipase C (PI-PLC) system (Cheng et al., 1991). The activation of GHSR1a in the NPY-expressing neurons of the Arc also increases their cytosolic Ca^{2+} concentration. This is mediated by N-type calcium channels, activated by protein kinase A (PKA). Ghrelin receptor can thus be coupled to protein G_s (Kohno et al., 2003). This Ca^{2+} signaling by ghrelin receptor activation leads to a release of growth hormone in the case of pituitary gland cells (Cheng et al., 1991), increased neural activity in NPY neurons (Cowley et al., 2003a), and alterations in the secretion of insulin by pancreatic cells (Date et al., 2002; Dezaki et al., 2004).

Ghrelin receptor activation in the hypothalamus also increases AMPK activity, which is connected to an increase of food intake (Andersson et al., 2004). The molecular mechanism behind this effect was further clarified by Adrews and colleagues (Andrews et al., 2008). Ghrelin receptor activation in cells co-expressing neuropeptide Y and agouti-related protein (NPY/AgRP) alters mitochondrial respiration in a manner dependent on uncoupling protein 2 (UCP2). Ghrelin produces an UCP2-dependent increase in cell firing in NPY/AgRP cells, an increase in mini inhibitory post-synaptic currents and a decrease in asymmetric putatively excitatory synapses in POMC expressing cells. The authors gathered evidence that indicates that the food intake increase produced by high AMPK activity requires UCP2.

PI3K activity and Akt (also known as protein kinase B) phosphorylation was shown to be mainly relevant for the effects of ghrelin in tissues other than the brain. Activation of GHSR1a on preadipocytes promotes their proliferation and differentiation through signaling involving the activation the ERK1/2 (a MAPK) and the PI3K/Akt pathway (Kim et al., 2004). In the brain, by co-administering ghrelin with an inhibitor of the PI3K pathway, Kanoski and colleagues demonstrated that this pathway is necessary for the increases in food intake produced by ghrelin administration on the ventral hippocampus (Kanoski et al., 2013).

4.4 Ghrelin enhances memory

Ghrelin has a well described effect on memory and learning. It was first observed by Carlini and colleagues that ghrelin ICV injected in rats increases latency time in the step-down test, a manifestation of increased memory retention (Carlini et al., 2002a). The authors further scrutinized this effect and found that the direct injection on the hippocampus produced this effect and that ghrelin did not affect memory retrieval (Carlini et al., 2010). Only long-term memory of the stepdown avoidance task was improved (Carlini et al., 2010). Dorsal hippocampal injections of ghrelin in rats produced an improvement in spatial memory as assessed by Morris water maze (Chen et al., 2011). This process is physiologically relevant since ghrelin found in the plasma crosses the blood-brain barrier and binds to

hippocampal neurons (Diano et al., 2006b). Diano and colleagues demonstrated that ICV-injected ghrelin dose-dependently increases retention in the T-maze footshock avoidance task and replicated the observed effects on step-down passive avoidance (Diano et al., 2006a). More importantly, they observed that the knockout for the ghrelin peptide has memory impairment in the novel object recognition test and that long-term administration of ghrelin on these animals, through subcutaneous administration with minipumps, recovers their performance in the test. The oral administration of the non-peptide ghrelin receptor agonists, GSK894490A and CP-464709-18, improves the performance in the novel object recognition task and Morri's water maze (Atcha et al., 2009). Ghrelin receptor knockout shows decreased hippocampus-dependent spatial memory as assessed by the Morris water maze (Davis et al., 2011). Albarran-Zeckler and colleagues observed that the ghrelin receptor knockout shows faster habituation to novel environments and decreased contextual memory, as assessed by contextual fear conditioning (Albarran-Zeckler et al., 2012).

There are several mechanisms suggested to explain effect of ghrelin on memory and learning. Diano and colleagues observed that the ghrelin receptor is highly expressed in all the regions of the hippocampus, promotes a process similar to LTP and increases synaptic spine density in the hippocampus (Diano et al., 2006b). Knockout mice for ghrelin have lower spine density and this phenotype is rescued to control levels through ghrelin administration (Diano et al., 2006b). This is consistent with the observation that treatment of organotypic slice cultures of hippocampus with ghrelin increase the amount of F-actin clusters in the CA1 (Berrout and Isokawa, 2012). These increases were not observed if cultures were treated with ghrelin receptor antagonists and were reverted if ghrelin-free media was used for 22h after ghrelin treatment (Berrout and Isokawa, 2012).

Diano and co-workers observed that acute hippocampal slices treated with ghrelin show a persistent increase in the slope of CA1 excitatory postsynaptic potentials after a 10-Hz stimulation (Diano et al., 2006b). Although this stimulation protocol is not recognized as an electrical protocol for synaptic plasticity in the hippocampus (Albensi et al., 2007), this result points towards an effect of ghrelin on long-term potentiation (LTP). LTP is considered a substrate for activity-dependent plasticity in the hippocampus, and an important component of spatial memory and memory retention (Bohme et al., 1993; Yu et al., 2017). Several studies subsequently showed the effects of ghrelin on LTP. Chen and colleagues showed that *in vivo* high frequency stimulation (HFS) LTP is prolonged by ghrelin and that this effects depends on MAPK activation by GHSR1a (Chen et al., 2011). Carlini and colleagues also showed that ghrelin reduces the threshold for HFS LTP induction in the CA1 of acute hippocampal slices, by a process dependent on the activation of nitric oxide synthase (NOS) (Carlini et al., 2010).

The work of Carlini and colleagues also suggested that the interaction between ghrelin and the serotonergic system may be involved in this effect. Ghrelin-mediated increases in memory retention can be prevented by direct hippocampal administration of the serotonin reuptake inhibitor fluoxetine before the trials (Carlini et al., 2007). Moreover, ghrelin inhibits secretion of serotonin in the CA1 of hippocampal slices of animals trained in the step-down test (Ghersi et al., 2011). As mentioned above,

NOS activation mediates ghrelin effects on LTP (Carlini et al., 2010). In the same study, it was observed that inhibition of NOS before the step-down test prevents ghrelin-mediated gains in retention. This observation is linked to the evidences regarding serotonin since NOS inhibitors are known to induce increases in hippocampal serotonin levels (Wegener et al., 2000), and increases in serotonin are described to impair LTP (Kim et al., 2006).

Chen and colleagues also observed that ghrelin increases the excitability at the presynaptic and postsynaptic level of afferent synapses on the dentate gyrus of the hippocampus (Chen et al., 2011). This effect was shown to be dependent on signaling through PI3K pathway. Inhibition of PI3K is able to prevent ghrelin-mediated improvements in memory (Chen et al., 2011). Ghrelin increases the activation of protein kinase A and subsequent increase in the phosphorylation of the NMDA receptor GluN1, in the hippocampus (Cuellar and Isokawa, 2011). This process is also a potential mechanism for the enhancement of memory. The activation of this pathway requires that GHSR1a either couples G_s protein directly or amplifies the G_s signaling of other GPCRs through heterodimerization, as mentioned above. Ghrelin also affects hippocampal neurogenesis, which is also involved in memory. The knockout mice for the ghrelin peptide has reduced numbers of progenitor cells in the subgranular region of the hippocampus (Li et al., 2013a). This reduction is reverted by exogenous administration of ghrelin. This reversion is accompanied by the correction of deficits in the performance of Y-maze and novel object recognition (Li et al., 2013b).

Regions other than the hippocampus may also be involved in the effects of ghrelin on memory (Carlini et al., 2004a). Carlini showed that ghrelin injections into the amygdala and dorsal raphe nucleus also improved memory. Nevertheless, in this work the hippocampus remained the most responsive region (Carlini et al., 2004a).

Ribeiro and colleagues observed that ghrelin receptor activation increases the surface and synaptic expression of GluA1 AMPA receptor and enhances NMDA-dependent LTP in the hippocampus (Ribeiro et al., 2014). Treatment of neuron cell cultures with the ghrelin receptor agonist MK-0677 for 1h before chemical LTP increased the AMPA receptor GluA1 membrane surface and synaptic expression. We also observed that these effects required the activation of the PI3K, PKA and PKC intracellular signaling pathways. Ghrelin agonist treatment also increased GluA1 phosphorylation at serine 831 and 845 and stargazing phosphorylation at serine 328/240. The ghrelin receptor purified with synaptosomal fractions of adult rat hippocampus, which is indicative of its expression in the vicinity of excitatory glutamatergic synapses (Ribeiro et al., 2014).

This effect of GHSR1a on learning and memory is believed to provide an evolutionary advantage, so that organisms are better capable to better remembering the location and condition of foraged foods (Moran and Gao, 2006)

4.5 Other roles and sites of action of ghrelin

Apart from the Arc, ghrelin also produces its orexigenic effects by acting directly on the VMH, PVN and LH of the hypothalamus (Lamont et al., 2012; Lopez et al., 2008; Mano-Otagiri et al., 2009). The PVN also receives fibers from ghrelin-expressing hypothalamic neurons (Cowley et al., 2003a). The area postrema and nucleus of the solitary tract have ghrelin receptors and intraventricular injections of the peptide increases Fos expression in these regions (Lawrence et al., 2002).

Ghrelin plays a role in the regulation of fat mass. In rodent, the weight increase induced by ghrelin is derived from an increase in fat mass and reduction of fat utilization (Tschop et al., 2000). Certain types of obesity are linked to ghrelin, as in the case of the Prade-Willi syndrome (Hillman et al., 2011). In this syndrome patients have elevated levels of plasmatic ghrelin and persistent hyperphagia. Pharmacologic modulation of ghrelin shows promise as a treatment for obesity. Antagonism of GHSR1a or reduction of ghrelin reduce food intake and body weight in diet-induced obese mice (Esler et al., 2007; Rudolph et al., 2007).

Ghrelin is also implicated in anxiety and depression. Intracerebroventricular and intraperitoneal injections of ghrelin induce anxiety-like behavior in mice and rats as assessed by elevated plus maze (Asakawa et al., 2001; Carlini et al., 2002b; Kajbaf et al., 2012). This effect is also elicited by direct injection into several nuclei of the hypothalamus (Currie et al., 2012), and is believed to be induced by the activation of the hypothalamic-pituitary-adrenaline axis, since intraperitoneal injections of ghrelin increase hypothalamic expression of CRH (Asakawa et al., 2001), and also due to the activation of the serotonergic system, since ghrelin produces a potent anxiogenic effect when injected into the dorsal raphe nucleus (Carlini et al., 2004a). There are also reports that ghrelin decreases anxiety (Alvarez-Crespo et al., 2012; Lutter et al., 2008; Spencer et al., 2012) and has an anti-depressive effect (Lutter et al., 2008). These observations remain to be reconciled. The anti-depressive effects of ghrelin may be explained by its promotion of hippocampal neurogenesis (Li et al., 2013a; Moon et al., 2009), which is thought to underlie the therapeutic action of several antidepressants (Sahay and Hen, 2007), and the immunosupressive effect it exerts through inhibition of proinflammatory cytokines on immunocytes (Himmerich and Sheldrick, 2010), also throught to have antidepressant effects (Tyring et al., 2006).

Stress increases the circulating levels of ghrelin in rodents and humans (Schellekens et al., 2012). Plasmatic levels and gastric mRNA for ghrelin are increased in rodents subjected to tail pinch and water avoidance stress (Asakawa et al., 2001; Kristenssson et al., 2006). In humans, the standardized Trier-Social-Stress test also increases plasmatic ghrelin levels in a manner correlated with cortisol levels (Rouach et al., 2007). Perhaps this effect explains the phenomenon of “comfort eating” during stress.

The mesocorticolimbic reward pathways are sensitive to ghrelin. The action of ghrelin on these regions explain its effect on hedonic food intake. GHSR1a is highly expressed in the VTA (Schellekens et al., 2012) and infusions of ghrelin at this region leads to the release of dopamine within the nucleus

accumbens (Jerlhag et al., 2007). Projections from the VTA to the nucleus accumbens and prefrontal cortex are important for food anticipation and food-seeking behavior (Schellekens et al., 2012). Ghrelin injections directly into the VTA enhances the behavior rats will perform in order to obtain a sucrose reward (Skibicka et al., 2012). Moreover, the administration of ghrelin in rats lowers the minimum dose of cocaine necessary to induce place preference (Davis et al., 2007). In humans, the activity in brain reward centers elicited by appealing food pictures is increased by the administration of ghrelin (Malik et al., 2008), and ghrelin is believed to be implicated in the mechanism of alcohol craving (Addolorato et al., 2006).

4.6 Constitutive activity of the ghrelin receptor

The ghrelin receptor has high constitutive activity (Holst et al., 2004). Constitutive activity on GPCRs can be defined as the ability of receptors to undergo agonist-independent isomerization from an inactive to an active state (Seifert and Wenzel-Seifert, 2002). This type of activity is not uncommon among GPCRs. By 2007, 60 wild type GPCRs were known to have constitutive activity (Smit et al., 2007). The constitutive activity of certain GPCRS, and its regulation by endogenous inverse agonists, is known to play an important role in homeostasis. This is the case for histamine H_3 receptor, important for arousal, attention and memory formation (Morisset et al., 2000) and melanocortin MCR_1 and MCR_4 receptors, important for pigmentation and body weight regulation (Adan, 2006). Moreover, many common GPCR antagonists with important clinical applications are now recognized to be inverse agonists of their respective receptors. Such is the case of the angiotensin AT_1 receptor antagonists losartan (Takezako et al., 2004), or the dopamine D_2 receptor antagonist haloperidol (Hall and Strange, 1997).

Constitutive activity of the ghrelin receptor was first described by Holst and colleagues (Holst et al., 2004). Just by being present on the membrane surface and in the absence of ligands, the ghrelin receptor produces intracellular signaling to the extent of nearly 50% of its activity while fully activated by agonists (Holst et al., 2003b). This type of activity is clinically relevant. The naturally occurring mutation of GHSR1A A204E (Pantel et al., 2006a) and F279L (Wang et al., 2004) have no constitutive activity but maintain agonist-induced activity. Individuals with these mutation have characteristic short stature. The phenotype suggests that the constitutive activity of ghrelin is important for growth hormone axis (Holst and Schwartz, 2006). Surprisingly both studies do not directly associate the mutations with alterations in appetite and body weight.

Nevertheless, through continuous ICV injection of the inverse agonist [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-substance P (SP), using osmotic pumps, Petersen and colleagues observed that the blockage of constitutive activity of the ghrelin receptor decrease food intake and body weight (Petersen et al., 2009). In the hypothalamus, blocking the constitutive activity led to a decrease in the expression of NPY and UCP2 and a decrease in the activation of CREB, potentially explaining the effects in food intake. ICV administration of the inverse agonist hexapeptide K-(D-1-Nal)-FwLL-NH2 also decreased fasting-induced hyperphagia (Fernandez et al., 2018). The gavage administration of a newly developed inverse agonist by AstraZeneca, capable of crossing the blood brain barrier, also induced a reduction in feeding

(McCoull et al., 2014). It was observed that the inverse agonist SP increases the threshold for pilocarpine induced convulsions in the hippocampus, in a manner similar to what is observed for GHSR1a KO (Portelli et al., 2012).

The constitutive activity of the ghrelin receptor activates signaling pathways distinct from those observed in agonist mediated activation. Soto and colleagues showed that both agonist and constitutive activity of the ghrelin receptor reduced Cav2 channel currents in hypothalamic neurons, and that this effect is mediated by G_q and $G_{i/o}$ -dependent mechanisms for each type of activity respectively. These channels modulate the presynaptic release of GABA on the hypothalamic neurons, linking ghrelin receptor activity with decreased inhibition (Lopez Soto et al., 2015). Of note, Holst and colleagues observed that the ghrelin receptor is constitutively internalized and this process is prevented by the inverse agonists SP (Holst et al., 2004).

Recently, Ge and colleagues showed that an already known peptide naturally secreted by the liver, the liver-expressed antimicrobial peptide 2 (LEAP2), is an endogenous antagonist of the ghrelin receptor (Ge et al., 2018b). The expression of this peptide is remarkably high following vertical sleeve gastrectomy surgery in mice. Blocking LEAP2 activity using antibodies increases production of growth hormone during fasting and administration of the peptide decreased food intake. M'Kadmi and colleagues found that this peptide also has inverse agonist activity (M'Kadmi et al., 2019). By characterizing the activity of the peptide on the ghrelin receptor they found that it has both inverse agonist and competitive antagonist activity, and that it inhibits the production of inositol phosphate, which the receptor induces under basal conditions and when stimulated by ghrelin. These works showed that the organism has the capacity to directly regulate the constitutive activity of the ghrelin receptor. Constitutive activity may also be regulated intracellularly by expression of G proteins or other downstream signaling molecules (Seifert and Wenzel-Seifert, 2002), but these mechanisms are yet to be described for the ghrelin receptor.

Kim and colleagues described that the ghrelin receptor expression in the hypothalamus increases dramatically within 48h of fasting (Kim et al., 2003). Since the cumulative constitutive activity of the receptor scales with the number of receptors expressed, the regulation of the number of receptors in the membrane indirectly regulates the amount of constitutive activity. Moreover, the phenotype of the ghrelin receptor knockout also models the effect of the absence of constitutive activity (Albarran-Zeckler et al., 2012; Davis et al., 2011). This suggests that the known effects of the knockout of the ghrelin receptor on memory and learning may be, at least in part, explained by the absence of this type of constitutive activity.

The link between the activity of ghrelin receptor and memory suggests that the constitutive activity of the receptor may also play a role. In this work we aim at clarifying whether and by what mechanisms the constitutive activity of the ghrelin receptor is involved in the learning and memory. We explore this part of the work in Chapter 2 of this thesis.

5 Social behavior

Social behavior can be defined as any interaction or type of communication between conspecifics of the same species (Chen and Hong, 2018). Social behavior is essential for the survival and fitness of species. Examples of essential types of social behavior are mating, parenting, aggressive and cooperative behaviors (Chen and Hong, 2018). There are a number of characteristics that makes social behavior much more complex than other types of behavior. Social behavior depends on many different types of sensorial cues. For instance, the male mice produces the pheromone ESP1 which regulates female reproductive behavior by activating the receptor V2Rp5 in the vomeronasal organ (Haga et al., 2010). In female mice the normal function of this organ is essential for maintenance of normal female-specific behavior. Complex ultrasonic vocalizations are another important pathway of communication, which enables rodents to signal facilitation or inhibition of social behavior (Portfors, 2007). Another characteristic is the fact that decision making during social interactions is happening between two agents that do not share internal states or previous experiences, which creates an irreducible level of uncertainty to the outcome of the decision (Chen and Hong, 2018). Finally, the outcome of social interaction changes over time, in part due to the dynamic nature of the internal states that influence social behavior. For instance, the VMH contains neuronal populations that when activated can induce aggressive or reproductive behavior (Anderson, 2016). It is theorized that this region integrates sensory information over time, namely olfactory, and influence the decision towards one type of behavior or the other.

Indeed, social and feeding behaviors are closely linked. For example, individuals must regulate their feeding behavior in order not to completely consume foraged nutrients to be distributed to their offspring and other individuals in the social group (Chen and Baram, 2016). Also, organisms change their social behavior according to metabolic state (Boyle et al., 2017). It is thus expected that the regulation of social behavior involves similar brain regions and neuropeptides as the regulation of feeding and foraging (Chen and Baram, 2016). Parenting is an aspect of social behavior that is closely associated with feeding. Organisms must either inhibit feeding in order not to consume the foraged food or even their offspring or increase feeding in order to acquire enough nutrients for the burdening activities of taking care of their progeny.

Galanin, an orexigenic neuropeptide, is produced by neurons in the medial preoptic area of the hypothalamus. These neurons are specifically activate during parenting, in males and females, and their optogenetic activation increases pup-grooming in males and prevents aggression towards pups and other males (Wu et al., 2014). It was also observed that the peripheral administration of urocortin II enhances parental behavior (Samuel et al., 2008). Hypothalamic levels of NPY and AgRP are increased during lactation, and acute suckling enhances the expression of NPY in the Arc and the dorsomedial hypothalamus (Li et al., 1998) (Chen et al., 1999). As for other types of social behavior, NPY receptor homologues in *C. elegans* confer differences in the sociability of these organisms. This species habitually aggregates when food is limited and become solitary when it is abundant. The two isoforms of the homologue receptor NPR-1 215F and NPR-1 215V are exclusively found on the social

and solitary strains of *C. elegans*, respectively. Causality was established by developing a transgene from of the strain that originally manifested social behavior but now expressed the solitary homologue NPR-1 215 V. These organisms subsequently developed solitary behavior (de Bono and Bargmann, 1998a).

Neuropeptide regulation of social behavior has been studied in pair-bonding, social cognition, social memory and social aggression. These categories of social behavior are particularly associated with two neuropeptides: oxytocin and vasopressin. These neuropeptides have both 9 aminoacids (diverging from each other at two positions) and are released into the periphery and inside the brain. They are produced each by a distinct neuron population, and both are found in high abundance in the supraoptic nucleus and PVN (Landgraf and Neumann, 2004). These neurons project either to the pituitary gland, which secretes the neuropeptide to the bloodstream, or to various brain regions. In terms of function, oxytocin is more associated with female sexual behavior such as sexual intercourse, parturition, lactation, maternal attachment and pair-bonding (Valtcheva and Froemke, 2019). Vasopressin is more associated with male sexual behavior such as erection and ejaculation, and also with aggression, territoriality and pair-bonding (Donaldson and Young, 2008).

As an example of the importance of oxytocin, it was observed that oxytocin increases the salience of perceived pup calls in mice, through a regulation of the balance between neuronal excitation and inhibition. This occurs specifically in the left auditory cortex and under optogenetic control of oxytocin neurons. This effect enables the successful retrieval of pups by mothers (Marlin et al., 2015). In the case of vasopressin, its receptor V1a (V1aR) is associated with pair-bonding. The two species of voles, praire voles (*Microtus ochrogaster*) and meadow voles (*Microtus pennsylvanicus*), are respectively monogamous and polygamous, and show different levels of expression of V1aR in the ventral pallidum, a region of the striatum. In the case of the polygamous species, a lower expression level, and the monogamous, a higher expression level. Through viral injection, increasing the expression of V1aR in the ventral pallidum of the prairie vole was associated with a remarkable change in behavior from polygamy into monogamy (Lim et al., 2004).

5.1 Social dominance hierarchies

Groups of organisms naturally form dominance hierarchies in which the dominance or submissiveness of each individual determines its rank and, therefore, access to limited resources such as food, shelter and reproductive partners. While individuals compete for rank there is learning and updating on status via interactions between individuals in the group (Qu et al., 2017). Social dominance hierarchies are pervasive in the animal kingdom, being used from insects such as ants, to fish or primates (Grosenick et al., 2007; Watanabe and Yamamoto, 2015; Wilson, 2000). This organization is postulated to be conserved among species since it prevents and hastens the resolution of conflicts between individuals, while serves as a mechanism that facilitates sharing of resources (Wang et al., 2014).

The first scientific assessment of social dominance hierarchies was performed by Thorleif Schjelderup-Ebbe (Schjelderup-Ebbe, 1922). Ebbe described how a group of domestic fowls manifested a “pecking order” according to which each member of the group had a certain priority in the access to food. This was a manifestation of their position in the social dominance hierarchy. Many behavioral paradigms have been developed to evaluate social hierarchy of rodents on a laboratory setting: the tube test (Lindzey et al., 1961), the social interaction test (Coura et al., 2013), the urine-marking test (Drickamer, 2001), the food competition test (Fredericson, 1950), the visible burrow system (Blanchard et al., 1995), the dominant-submissive relationship paradigm (Feder et al., 2010), the resident intruder test (Kaliste-Korhonen and Eskola, 2000), and the labor division test (Mowrer, 1940).

More than an important and ubiquitous biological phenomenon to study, social dominance hierarchies are of clinical relevance, since a link has been proposed between heightened dominance motivation in humans and psychopathology (such as externalizing disorders, narcissistic traits and mania proneness) (Johnson et al., 2012), and the depression and anxiety-like symptoms experienced after repeated social defeat (Qu et al., 2017).

Several brain regions and molecules are suggested to play an important role in the establishment and maintenance of social dominance hierarchies.

5.2 Brain regions involved in social dominance

There are strong indications that in primates the orbitofrontal cortex codes the social dominance of other individuals (Watson and Platt, 2012) (Azzi et al., 2012). In primates, the anterior and dorsal prefrontal cortex and midsuperior temporal sulcus are also involved in the perception of social status, along with a number of subcortical regions such as the amygdala, the proximity of the raphe nucleus, reticular formation, hypothalamus and septum. The gray matter volume of these subcortical regions correlate with social status, in various manners (Noonan et al., 2014).

The prefrontal cortex is a promising region for several reasons. It was observed that in primates, during social interaction, the activity of prefrontal neurons was increased for the dominant animal, while it was

decreased for the submissive one (Fujii et al., 2009). In humans, the dorsolateral prefrontal cortex shows specific differential activity according to social hierarchy status (Zink et al., 2008a). In rodents, lesions to the prefrontal cortex region anterior cingulate lead specifically to decreased interest in other animals and reduced memory of social partners (Rudebeck et al., 2007). Wang and colleagues were able to increase the rank of subordinate mice by controlling the neural activity of the medial prefrontal cortex (Wang et al., 2011a). The authors used viral injections in order to induce high expression of the AMPA receptor GluR4. In basal conditions, an increase in mEPSC amplitude on layer V pyramidal neurons was observed in dominants animals when compared to submissive ones. The authors also observed a specific increase in c-fos expression in the prelimbic cortex of dominant mice after tube test encounters. *In vivo*, the authors subsequently observed that the medial prefrontal cortex is particularly activate during push and resistance and not retreat, during the tube test (Zhou et al., 2017). The acute inhibition of the dorsal dmPFC *in vivo,* using DREADD, also decreased the effortful behavior leading to more losses, while acute activation of dmPFC *in vivo* using optogenetics, increased effortful behavior and led to more wins. These works used behavioral test for social dominance hierarchy in the tube test, a simple assay that is reliable and consistent with other measurements of social dominance hierarchy, such as, territory urine marking and courtship ultrasound vocalization (Fan et al., 2019).

These changes in dorsal medial prefrontal cortex suggests that connected brain regions may also take part in social dominance hierarchy processing. The authors verified that optogenetic stimulation in the vicinity of the dmPFC, namely in infralimbic and the posterior part of the anterior cingulate cortex, does not produce these effects (Zhou et al., 2017). However, the specific activation of efferent fibers from the mediodorsal thalamus increased the hierarchy rank of submissive mice, in a manner sustained after the end of the stimulation protocol (Zhou et al., 2017).

Nucleus accumbens is a brain region of the ventral striatum that receives projections from the prefrontal cortex. This region is known to be involved in the motivation to compete in social contexts in humans (Le Bouc and Pessiglione, 2013) and its disruption affects dominance behavior in rats (Fantin and Bottecchia, 1984). During aggressive confrontations with more dominant individuals, rats manifest an increased phasic dopamine release from the VTA into the NAcc (Anstrom et al., 2009). In general social behavior, activity on projections from VTA to NAcc can predict bouts of social behavior and manipulation of the activity of these fibers, through optogenetics, stimulates this behavior (Gunaydin et al., 2014).

The susceptibility to stress and anxiety has an important negative impact in self-confidence and competitiveness of humans (Goette et al., 2015). This is also the case in rodents, and it was recently found that more anxious rats, in comparison with less anxious more dominant counterparts, have reduced mitochondrial complex I and II proteins, ATP and respiratory capacity and higher ROS production in the NAcc (Hollis et al., 2015). Moreover, pharmacologic inhibition of these mitochondrial proteins reduces the social ranks of individuals and enhancement of metabolism in this region reduces the submissiveness of highly anxious individuals. More recently, it was observed that the diazepam-mediated benefits for social dominance are linked to its effect of increasing the dopamine release from

VTA to the NAcc and increasing mitochondrial respiration on the NAcc (van der Kooij et al., 2018). Diazepam disinhibits VTA dopaminergic neurons and the activation of D1 dopamine receptors on the NAcc is linked to the increase in mitochondrial respiration and increase in ATP levels. Despite this body of work, the neurophysiology of the NAcc in social dominance hierarchy formation and maintenance still remains to be clarified.

5.3 Molecular determinants of social dominance

There are already several molecules and pathways known to regulate this type of behavior. In humans, basal plasmatic testosterone is able to predict dominant and antisocial behavior. Upon a competitive challenge, testosterone levels increase with the anticipation of competition and rise the highest in winners and decline in losers (Mazur and Booth, 1998). These surges in plasmastic testosterone were observed in wrestling matches (Fry et al., 2011). In castrated cats, administration of testosterone or estradiol into the preoptic area of the hypothalamus leads to the manifestation of some dominant behaviors (Bean and Conner, 1978).

Neuropeptides are known to have a role in this type of behavior. In mice, removing an alpha male from an already established hierarchy leads to an increase in aggressiveness among the remaining individuals and to the establishment of a new dominance hierarchy. With this new hierarchy, there is an increase in the expression of gonadotropin-releasing hormone (GnRH) in the medial preoptic area of both dominant and submissive animals, but only the new dominant alpha show an increase in plasmatic testosterone (Williamson et al., 2017).

There is also a connection between stress and social hierarchy. Among rats, a first encounter between two males leads to the formation of a sustained hierarchy (>1 week) only if one of the two males is stressed (Timmer et al., 2011). Timmer and colleagues observed that in these types of encounters the dominant male had a higher expression of oxytocin receptor mRNA in the medial amygdala 3h after the encounter, with no change in the vasopressin receptor V1aR expression. No differences were observed in the lateral septum. Consistent with these observations, knockouts for oxytocin receptor show decreased aggressiveness and dominant behavior (Lazzari et al., 2013), although their generalized disinterest in social interaction can be a confounder. Using the VBS model, it was observed that submissive animals have higher levels of plasmatic corticosterone, which by itself can contribute to an inhibition of the production of testosterone by Leydig cells (Hardy et al., 2002). At different timepoints during the experiment, lower levels of testosterone and luteinizing hormone (an hypothalamic-released enhancer of Leydig cells steroidogenesis) were observed in the submissive animals. These results suggest not only a direct effect of stress on peripheral tissue, but also on the hypothalamus. Timmer and Sandi also observed that corticosterone is linked to the maintenance of social hierarchy (Timmer and Sandi, 2010). If animals are administered with cortisol before the first encounter, and if the injected animal becomes dominant, the formed hierarchy will be maintained for more than 1 week. After the first encounter, if animals are injected with cortisol, a long-term hierarchy will only be formed if the animal injected was submissive. Using the VBS paradigm, Makinson and colleagues found that social

subordinate rats had decreased expression of glutamic acid decarboxylase (GAD) 67 in the PVN and the interfascicular nucleus of the bed nucleus of stria terminalis (BNST) and increased expression of this protein in the hippocampus, medial prefrontal cortex, and dorsal medial hypothalamus (Makinson et al., 2015). BDNF expression was also specifically increased in the BNST. The BNST is an important brain region for the processing of stress, anxiety and appetitive/aversive behavior (Ch'ng et al., 2018). This increase in GAD 67 expression suggests increased inhibition on these regions, given the importance of this protein for the production of the inhibitory neurotransmitter GABA. This patchwork of data shows that stress plays an important role in the formation and maintenance of social dominance hierarchies, even though it is still impossible to delineate a clear model for the relationship between the two.

The serotonergic neuromodulator system is also known to be involved in social hierarchy (Wang et al., 2014). This connection is well described for crustaceans, like the crayfish (Edwards and Kravitz, 1997). For instance, injections of serotonin into crayfish increases the willingness of subordinate animals to engage in confrontations with more dominant crabs (Huber et al., 1997). In monkeys, the administration of SSRIs reduces aggressive behavior and increases affiliation which contributes to increasing the social rank of individuals (Raleigh et al., 1991). In mice, chronic reduction of serotonin neuron firing, through conditional overexpression of Htr1a, increases aggression (Audero et al., 2013). Homozygous knockouts for serotonin transporter 5-HTT also show decreased dominant behavior when battling wild-type animals (Lewejohann et al., 2010). The noradrenaline and acetylcholine system also play a role. The knockouts for the acetylcholine receptor beta 2 nAChR have reduced social dominance. This is prevented by a depletion of noradrenaline in the prelimbic cortex (Coura et al., 2013).

Regarding the genetics of social dominance, animals can be selectively bred according to dominant and subordinate behavior and this behavioral profile is rapidly enhanced after only a few generations (Masur and Benedito, 1974; Moore et al., 2002). In a specific line, these traits were found to be explained in part by an upregulation in the expression of the synapsin IIb (SynIIb) gene in the hippocampus and striatum in subordinate mice when compared to dominant ones. These alterations were absent from the prefrontal cortex. Knockout studies have also demonstrated that synapsin II and III are involved in social dominance (Greco et al., 2013b). The acute administration of an SSRI was able to reduce the expression of synapsin IIb in the hippocampus and reduce submissive behavior (Nesher et al., 2015b). A study on the genetic expression profile differences along the social dominance hierarchy in rainbow trouts identified that the expression of the ependymin gene correlates with social hierarchy rank (Sneddon et al., 2011). This is a glycoprotein described to be involved in memory formation and synaptic plasticity in the hippocampus (Shashoua, 1991). Inactivation of this protein led to decreased competitive and aggressive behavior among more dominant trouts (Sneddon et al., 2011). Mice subjected to maternal deprivation display subordinance during adulthood and are known to have a distinct gene expression profile from those of control more dominant mice (Benner et al., 2014a): lower prelimbic cortex Map2 and hippocampus CA1 cfos expression, and higher basolateral amygdala and infralimbic cortex cfos expression (Benner et al., 2014a). A mutation in the synaptic protein

SHANK3 has also been associated with increases in social dominance as assessed by tube test (Zhou et al., 2016).

5.4 Early life stress

Adverse conditions experienced early in life induce changes in the brain function of adults (Everson-Rose et al., 2003). In humans these changes manifest in deficits in cognition, such as memory and executive function, and in affective functions, such as emotional regulation and processing of rewards (Pechtel and Pizzagalli, 2011). This is also the case for rodents, who experience increased anxiety (Dalle Molle et al., 2012), impaired social behavior (Raineki et al., 2012) and impaired memory and learning (Naninck et al., 2015). These adverse conditions are most prominently the result of abnormal maternal care (Bowlby, 1950; Chen and Baram, 2016). Early life maternal deprivation and social isolation was shown to decrease competitiveness of adult mice, in a competition for water consumption after a period of low availability of water (Benner et al., 2014a).

Early life stress affects brain structure and physiology in numerous ways. It was observed that in women, early life stress increased cortisol levels in childhood, and these levels predicted a decreased functional connectivity between amygdala and ventromedial prefrontal cortex in adulthood (Burghy et al., 2012). This connectivity is involved in emotional regulation and its perturbation is associated with anxiety and depression. ELS also increases spontaneous and mini EPSC frequency in CRF-expressing dorsal-medial neurons of the hypothalamus of neonatal mice (Gunn et al., 2013). In this region, the glutamate uptake by astrocytes was decreased. With increased maternal care, there is less CRF expression in the PVN, and the CRF-expressing neurons have a lower number of synapses onto them (Korosi et al., 2010). In mice that endured ELS, CRF expression is increased in the hippocampus, and excessive activation of CRF receptors causes dendritic atrophy and attenuation of LTP, as well as impairment in the performance of novel object recognition and morris water maze (Ivy et al., 2010).

ELS is known to impair dendritic development of pyramidal neurons in layers II/III and V of dorsal agranular cingulate cortex and prelimbic cortex (Yang et al., 2015). These changes in the cingulate cortex persist to adulthood. CRF receptor activation was again implicated in this phenotype and in impairment of prefrontal cortex-dependent cognitive tasks (Y-maze spontaneous alternation) (Yang et al., 2015). Repeated stress perturbs medial prefrontal cortex neuron spine morphology, and leads to overall shrinkage, which is connected with dysregulation of the hypothalamic-pituitary-adrenal axis and stress-related disorders (Radley et al., 2008). However, there is still some controversy in this field of study since work from Tada and colleagues showed that social separation increased social dominance (Tada et al., 2016b). Isolated juvenile rats manifested an inactivation of actin-depolymerizing factor (ADF)/cofilin in the medial prefrontal cortex. This increases the stability of actin in the spines and reduces the mobility of AMPA receptors, as assessed by chemical LTP on brain slices. Artificial activation of ADF/cofilin *in vivo* reverted the social dominance of these animals (Tada et al., 2016b).

6 NPY

NPY has an important role in the regulation of feeding behavior, in memory and in the modulation of social behaviors. As such this neuropeptide is well positioned to play a role in the intersection between important physiological aspects necessary for animal survival. Food deprived rats show increased NPY mRNA and protein expression in hypothalamic neurons. These levels return to normal once the animals are fed (Beck et al., 1990; Brady et al., 1990; Sahu et al., 1988). Moreover, NPY injections into the hypothalamus produces a quickly-starting long-lasting increase in feeding, an effect that does not decrease with prolonged NPY injection (Clark et al., 1984; Kalra et al., 1988).

NPY is a 36 aminoacid peptide structurally homologous to other peptides of the pancreatic poplypeptide family, namely pancreatic polypeptide and peptide YY in humans (Tatemoto et al., 1982). It is named after its C-terminal amidated tyrosine (Y). The human NPY gene is found on the small arm of the chromosome 7 (p15.3) and the transcript contains 4 exons and 3 introns, totaling 8000-base pairs (Dumont and Quirion, 2013). The processed transcript is translated into a 97 amino-acid pre-pro-NPY containing a signal sequence, that allows the targeting of the peptide to the endoplasmic reticulum in to be packed into secretory vesicles. Pre-pro-NPY also includes the mature peptide NPY, the amydation proteolytic site, and the carboxy-terminal extension CPON (which is a 30 amino-acid peptide, co-released with NPY, and with an yet unknown function) (Sandeva et al., 2007). The processed mature NPY is released from LGVs. NPY is one of the most highly expressed neuropeptide and can be widely found in the central and peripheral nervous system (Colmers and Wahlestedt, 1993). It is found in interneurons of the forebrain and projection neurons in regions like the Arc, lateral geniculate nucleus and nuclei of the brainstem. It colocalizes with other modulators and neurotransmitters such as norepinephrine, GABA, somatostatin and AgRP (Colmers and Wahlestedt, 1993). The hypothalamus expresses the highest levels of NPY. NPY neuron cell bodies are mostly found in the Arc and LH, and their neurites spread through these regions. The PVN is enervated by projecting NPY neurons from the brainstem and the Arc. NPY-expressing interneurons can be found in all cortical regions but are most prevalent in the cingulate and temporal cortex and in the lowest occipital lobe. The hippocampus also contains a high number of NPY-positive neurons that innervate locally but may also receive extrahippocampal projections (Colmers and Wahlestedt, 1993). In the case of the striatum, there is a high prevalence of NPY interneurons in the nucleus accumbens and bed nucleus of the stria terminalis. Remarkably, NPY is almost absent from the thalamus and cerebellum (Colmers and Wahlestedt, 1993). NPY can cross the blood brain barrier in a non-saturable manner (Kastin and Akerstrom, 1999).

The knockout mice for NPY manifests a decreased food intake after fasting, are more anxious than controls and have hypoalgesia in the hot plate test (Bannon et al., 2000a). This knockout has also increased bone mass (Baldock et al., 2009)

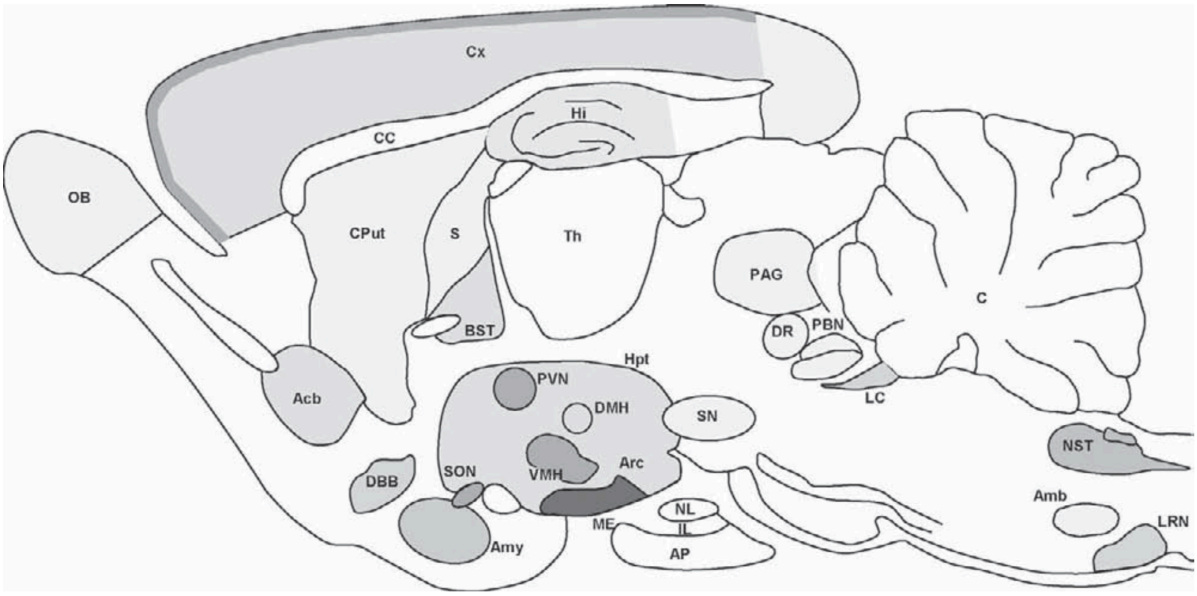


Figure 1.4 - Distribution of NPY mRNA in rat brain. OB, olfactory bulb; CPut, caudate and putamen; ME, median eminence; SN, substantia nigra; LRN, lateral reticulate nucleus; PAG, periaqueductal grey, DBB; diagonal band of broca; LC, locus coeruleus; Adapted from (Dumont and Quirion, 2013)

6.1 NPY Receptors

Mammals express 5 receptor subtypes for NPY: NPY1R, NPY2R, NPY4R, NPY5R and NPY6R. These are all GPCRs. These subtypes are expressed in mammalian brains with the exception of NPY6R, which although found on monkey and humans as mRNA, is only functional in a few species such as mouse and rabbit (Michel et al., 1998). The NPY receptors are remarkable in how different their sequence identity is from each other. NPY1R and NPY2R share only 31% of their sequence unlike any other peptide biding receptor. NPY4R is only 41% identical to NPY1R and was suggested to be one of the fastest evolving receptors with only 75% homology between humans and rat (Larhammar, 1996). In the central nervous system, NPY1R and NPY2R are the most abundant subtypes. Using radioactive isotope-marked peptide YY, a close homolog to the neuropeptide Y that binds its receptors, both rat and mouse NPY1R was found to be most expressed in the cortex, while NPY2R is most expressed in almost every other region, most prominently the hippocampus (Dumont et al., 1998). The hypothalamus expresses both types to an almost similar extent.

NPY receptors are coupled to Gi/o, which leads to a reduction of the production of cAMP (**Figure 1.5**). This is observed in almost every tissue observed (Michel et al., 1998). There are indications that activation of the NPY1R may also increase intracellular calcium levels, as observed in rat mesenteric small arteries (Prieto et al., 2000). In this case, the activation of NPY1R inhibited the effects of forskolin, an activator of adenylyl cyclase, further supporting the connection between activation of the NPY receptor and reduction of cAMP production. Activation of NPY receptors was also shown to stimulate the production of inositol triphosphate and diacylglycerol (Perney and Miller, 1989), and there are indications that it activates phospholipase A_2 (Martin and Patterson, 1989). In neurons, NPY1R, NPY2R

and NPY4R can activate GIRK class K^+ channels and inhibit presynaptic N- and P/Q-type voltage-gated Ca^{2+} channels (Ewald et al., 1988; Sun et al., 1998). This contributes to hyperpolarization of the cell membrane and inhibition of presynaptic neurotransmitter release. In the neocortex, NPY1R is expressed in all layers but most prominently in the superficial layers II and III (Larsen et al., 1993) (Leroux, 2002). In central amygdala, NPY1R is known to activate intracellular signaling that activates CaMKIV (Pandey, 2003) and in vascular smooth muscle cells, the NPY1R and NPY5R receptors activate the phospholipase C - PKC pathway – ERK pathway, and through increases of intracellular Ca^{2+} activates CaMKII (Pons et al., 2008).

The NPY1R knockout show increased weight, fat mass and plasmatic levels of leptin and glucose when on a high fat diet (Pedrazzini, 2004). Depending on the genetic background of the mouse models, the NPY2R knockout may manifest increased (C57BL/6) or decreased (mixed 129SvJ-C57BL/6 background) anxiety and depression (Zambello et al., 2011). Of note, yohimbine-mediated increases in dopamine in medial prefrontal cortex of control of congenic C57BL/6 were not observed in NPY2R knockouts with this background (Zambello et al., 2011)

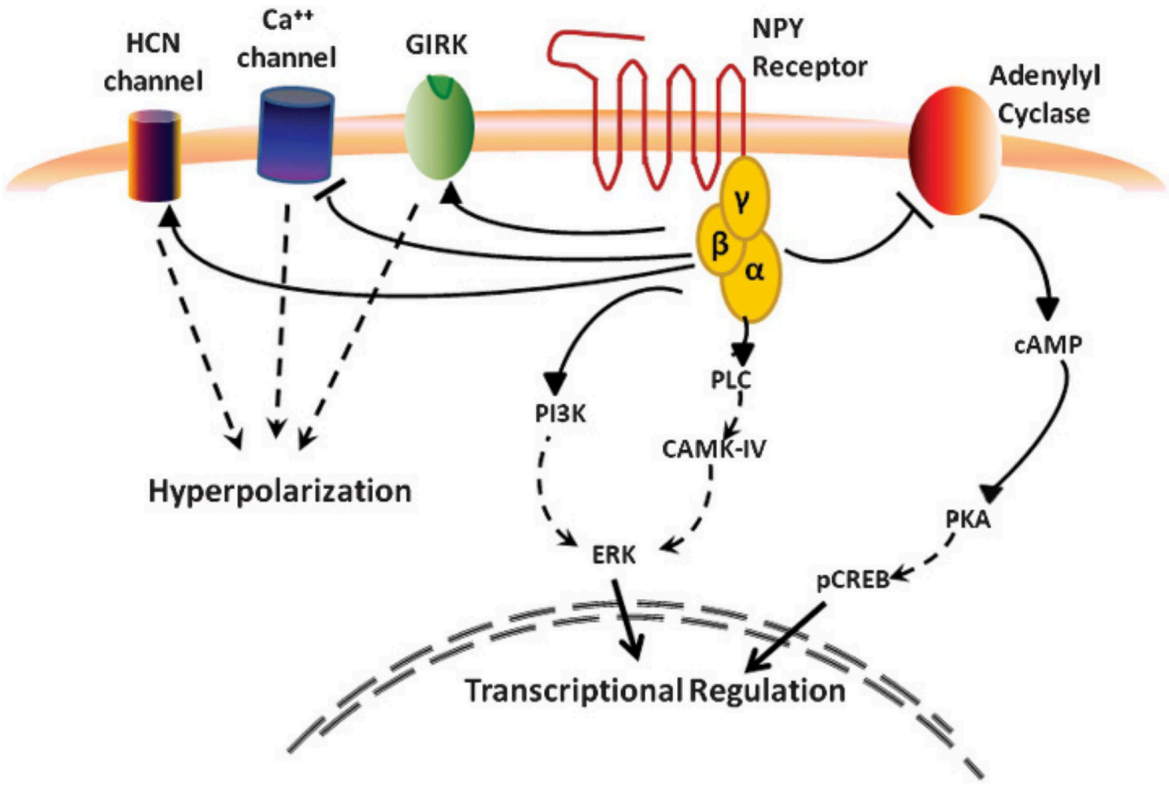


Figure 1.5 – Intracellular signaling of NPY receptors. HCN, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Adapted from (Sah and Geracioti, 2013)

6.2 The effects of NPY on electrophysiology

Activation of NPY1R and NPY2R is known to inhibit presynaptic GABA release and whole-cell calcium currents in suprachiasmatic neuron autaptic cultures (Chen and van den Pol, 1996). NPY is also known

to reduce evoked post-synaptic excitatory potentials on pyramidal cells in the central nervous system (Colmers and Bleakman, 1994; Klapstein and Colmers, 1993). NPY is considered an endogenous anticonvulsant. Excessive electrical activity leads to increased release of the peptide, which inhibits excitatory activity (Colmers and Bleakman, 1994). Indeed, the knockouts for NPY peptide show increased susceptibility to seizures (Erickson et al., 1996). In the hippocampus, this inhibition was found to be produced by the presynaptic activation of NPY2R and subsequent inhibition of N- and P/Q-type voltage-gated Ca^{2+} channels (Qian et al., 1997). Also in the hippocampus, NPY was found not to have an effect on postsynaptic inhibitory potentials (Klapstein and Colmers, 1993). In the hypothalamus, the activation of the NPY1R was shown to reduce spike frequency and hyperpolarize the membrane potential of hypocretin cells (Fu et al., 2004). This effect was again connected to the activation of GIRK currents. Moreover, the antagonism of NPY1R increased spontaneous firing of these cells, suggesting a continuous NPY-mediated inhibition. The authors observed a reduction in evoked EPSP amplitude and miniature EPSC frequency was reduced, with no change in amplitude of the latter. This is indicative of presynaptic effects of the NPY receptors, and through use of agonists, was linked to NPY1R, NPY2R and NPY5R. Inhibitory miniature IPSC were unaffected but there were indications that spontaneous IPSC frequency was reduced (Fu et al., 2004).

These effects on both the hypothalamus and hippocampus stand in contrast with the effects of NPY in the cortex. In cortical pyramidal neurons of layer V, while NPY also induces a prolonged depression of evoked EPSC amplitude, short duration exposure to NPY induces a prolonged increase in evoked IPSC amplitude (Bacci et al., 2002). The authors argue that these effects on inhibitory currents were the result of enhanced Ca^{2+}-dependent release of GABA, since under high extracellular K^{+}, NPY increased mIPSC frequency (without affecting amplitude), and this increase was reverted by Cd^{2+}, a Ca^{2+} channel blocker. In the case of cortical interneurons, IPSCs amplitude were reduced after a short exposure to NPY. These distinct effects, in comparison to other regions, may be explained by the prevalence of NPY1R in the cortex, while other NPY receptors are more highly expressed in the hippocampus and hypothalamus (Dumont et al., 1998). More recently this effect was replicated in layer V neurons of the infralimbic prefrontal cortex (Vollmer et al., 2016). Based on this evidence we can assume NPY1R and NPY2R can be found both at the pre and the postsynaptically. Nevertheless the synaptic effects of NPY are complex and not yet fully understood.

6.3 Effects of NPY on behavior

NPY and the activation of its receptors in the brain produce an overwhelming number of effects. They are known, for instance, to increase respiratory quotient, modulate locomotor activity, produce hypothermia, reduce sexual behavior, control seizures and shift the circadian cycle (Dumont et al., 1992; Gotzsche and Woldbye, 2016).

NPY is one of the strongest inducers of feeding, as observed by ICV and direct hypothalamic injection in rats (Clark et al., 1984; Stanley et al., 1985). Further evidences of the role of NPY on feeding are the fact that administration of NPY antibodies into the ventromedial and paraventricular hypothalamus

decrease feeding (Dube et al., 1994) and the attenuation of the release of NPY from the Arc, through the local expression of an antisense cRNA for NPY, significantly decreases food intake and weight in rats (Gardiner et al., 2005).

6.4 Effects of NPY on memory

NPY enhances memory and learning as assessed by ICV injections before testing in a T-maze footshock avoidance or step-down passive avoidance by mice (Flood et al., 1987). This effect is the result of NPY2R activation in a manner not linked to the increase of feeding induced by NPY (Flood and Morley, 1989). Furthermore, if a more intense training schedule is applied to treated mice, activation of the NPY2R induced a decrease in memory retention instead (Flood and Morley, 1989). A careful dissection of the effect of NPY and NPY antibodies on memory has shown a more complex relationship (Flood et al., 1989). While injections of NPY and NPY antibody into the dorsal hippocampus induce an enhancement and reduction of learning and memory, respectively, injections into the ventral hippocampus have the reverse effect. Injections into the septum produce similar effects to the dorsal hippocampus while injections into the amygdala produce effects similar to the ventral hippocampus. The knockout for NPY2R show deficits in memory assessed by the Morris water maze and reduced performance on the object recognition task (Redrobe et al., 2004). ICV injections of NPY prevented the expression of dorsal hippocampus dentate gyrus LTP (perforant path-granule cell synapses), assessed *in vivo* within 30 mins of the injections. By analysis of synaptosomes the authors verified that in animals treated with NPY, there was an increase in the activation of c-Jun N-terminal kinase (JNK) (Whittaker et al., 1999).

6.5 NPY and social behavior

Perhaps the most well described behavioral manifestations of the administration of NPY is its anti-anxiety and anti-stress effect. It was first observed by Heilig and co-workers that ICV injections of nanomolar doses of NPY produce an anxiolytic effect on the Montgomery conflict test and the Vogel drinking conflict test, two pharmacologically validated models of anxiety (Heilig et al., 1989). These results have been reproduced and validated numerous times (E. and M., 2004).

The connection between social behavior and NPY physiology has been studied over many dimensions. Activation of NPY1R specifically in the basolateral nucleus of the amygdala (BLA) increases social interaction in rats (Sajdyk et al., 1999). Also, perturbation of the noradrenaline-expressing projections of the locus coeruleus reduces social interaction, in a manner that is reversed by the ICV administration of NPY (Kask et al., 2000). NPY injections into the dorsocaudal septum also decrease anxiety in social interaction in rats (Kask et al., 2001), and this effect is mediated by the activation of NPY1R. NPY prevents a reduction in social interactions produced by urocortin injections into the BLA (Sajdyk et al., 2004). We can conclude NPY and NPY1R activation produces a pro-social effect, perhaps because of reduced anxiety. Similar experiments as those that measured the vasopressin receptor expression in prairie and meadow voles were conducted for the measurement of central NPY (using a NPY antibody)

(Hostetler et al., 2013), although no differences were observed in the ventral pallidum. Stress by overcrowding in mice induces an increases anxiety-like behaviors, accompanied by increased expression of NPY and NPY1R mRNA in the hypothalamus and increased levels of corticosterone (Lin et al., 2015).

Regarding the connection between NPY and social hierarchy the literature is sparse. Rats subjected to chronic stress, in the form of housing on a visible burrow system (VBS), developed alterations in feeding according to their social rank (Melhorn et al., 2010). During the starting phase of exposure to the stress and while hierarchies were being established, dominants and subordinates showed reduced meal frequency, and subordinates in particular showed a reduction in meal size and a greater decrease in body weight. After dominance hierarchies were established, dominants returned to a feeding pattern similar to controls while subordinates maintained a reduction in feeding. While recovering from the stress exposure, subordinates displayed an increased feeding. The stressed population showed an increased expression NPY mRNA in the Arc, with no differences between hierarchy positions. Plasmatic corticosterone levels were altered during VBS housing but only on subordinates.

Chapter 2 - Thesis objectives

The main aim of this work is to contribute for the elucidation of the role of the constitutive activity of the ghrelin receptor on memory and learning and the possible mechanisms involved. A second goals was to help elucidate the molecular and neurophysiologic basis of how the brain codes social hierarchy following a form of stress.

To achieve these goals, we performed the following tasks:

- Part 1 – Constitutive activity of the ghrelin receptor, learning and memory:
    - Determine whether pharmacologic manipulation of the constitutive activity of the receptor alters learning and memory
    - Assess if glutamate receptor expression, mobility and plasticity is affected by the constitutive activity
    - Assess the intracellular signaling involved on these effects
- Part 2 – Social dominance hierarchy, stress and NPY:
    - Determine whether an early life stress model produces consistent social dominance hierarchies
    - Assess the neurophysiologic correlates of social dominance hierarchy position
    - Assess and validate candidate genes that may be responsible for neurophysiologic correlates of social dominance hierarchy

Chapter 3 – The effects of ghrelin receptor constitutive activity on memory

The presented work is under preparation for submission as a research article:

Luís F. Ribeiro *, Tatiana Catarino *, Mário Carvalho *, Sandra D. Santos, Luísa Cortes, Patricio O. Opazo, Lyn Rosenbrier Ribeiro, Daniel Choquet, José A. Esteban, João Peça, Ana Luísa Carvalho, Constitutive ghrelin receptor activity supports memory formation and modulates hippocampal AMPA receptor traffic.

* These authors contributed equally to the work

I contributed to the design, execution and analysis of the behavioral experiments and the surgeries for intrahippocampal implantation of cannulas and behavioral experiments involving intrahippocampal injections. I also participated in the writing of the article.

1 Introduction

Ghrelin is a peptide hormone believed to signal meal initiation (Kojima et al., 1999; Nakazato et al., 2001) and is found at the highest concentration in human plasma immediately before each meal (Cummings et al., 2001). It is mainly secreted by X/A-like cells in the oxyntic glands of the stomach and intestine (Date et al., 2000). Apart from effects on food intake (Mason et al., 2014) and feeding behavior (Hsu et al., 2015; Kanoski et al., 2013), ghrelin influences several other physiologic systems. For example, it is well established that ghrelin improves learning and memory (Carlini et al., 2002b; Diano et al., 2006b), regulates reward through its action on the mesolimbic dopamine system (Abizaid et al., 2006), modulates anxiety- (Carlini et al., 2002b) and depressive-like (Lutter et al., 2008) behaviors, and affects long-term fear memory (Meyer et al., 2014a).

The actions of ghrelin are mediated by the growth hormone secretagogue receptor type 1a (GHSR-1a) G protein-coupled receptor (GPCR), whose activation by ghrelin regulates gene expression, neuronal excitability and AMPA receptor trafficking (Cowley et al., 2003a; Diano et al., 2006b; Ribeiro et al., 2014; Shi et al., 2013). In the brain, GHSR-1a is highly expressed in the hypothalamus, pituitary gland and hippocampus (Guan et al., 1997b) and its expression levels increase during fasting (Fernandez et al., 2018; Kim et al., 2003; Petersen et al., 2009). Interestingly, GHSR-1a displays unusually high constitutive activity, corresponding to approximately 50% of its maximal activity (Holst et al., 2003a), that results from a natural shift in the equilibrium between its inactive and active conformations, in the absence of ligand (Damian et al., 2015). The ligand-independent GHSR-1a activity plays a role in the control of food intake and regulation of body weight (Els et al., 2012; Fernandez et al., 2018; McCoull et al., 2014; Petersen et al., 2009), and in the acquisition of conditioned taste aversion (Li et al., 2018). Human mutations that lead to a selective loss of constitutive activity of GHSR-1a, but that do not interfere with ghrelin-induced activation, are associated with familial short stature (Inoue et al., 2011; Pantel et al., 2006b; Pantel et al., 2009). Mechanistically, the GHSR-1a constitutive activity has been shown to reduce presynaptic Ca_v2 currents and GABA release in hypothalamic and hippocampal neurons (Lopez Soto et al., 2015; Valentina et al., 2018), by reducing the cell surface expression of Ca_v2 channels (Mustafa et al., 2017). However, it is likely that other physiological processes and mechanisms are impacted by the ligand-independent activity of the ghrelin receptor (Mear et al., 2013).

Ghrelin receptor knock-out animals display spatial and contextual memory impairments (Albarran-Zeckler et al., 2012; Davis et al., 2011), which can be attributed to the absence of ghrelin-triggered effects, but also to the loss of ligand-independent activity. However, the physiological importance of GHSR-1a constitutive activity for learning and memory has not been described. Here, using a combination of behavior analysis, imaging, biochemical and electrophysiological approaches, we uncover a role for the constitutive activity of the ghrelin receptor in providing tonic control for the regulation of AMPA receptor traffic, influencing synaptic plasticity in the hippocampus and interfering with learning and memory *in vivo*.

2 Materials and Methods

2.1 Animals

For the behavior experiments, male C56BL/6 mice from Charles River were used. The animals were kept in the Animal Facility of the CNC/Faculty of Medicine of the University of Coimbra with access to food and water ad libitum. The environment was kept in temperature and humidity controlled conditions under a 12h dark-light cycle (light period 6h00-18h00). Animals used were 8-15 weeks old. Behavioral testing was reviewed and approved by the animal use and ethics committee (ORBEA) of the CNC/Faculty of Medicine, University of Coimbra, and by the Portuguese national authority for animal experimentation (DGAV), and all procedures were performed according to the guidelines of the DGAV and Directive 2010/63/EU of the European Parliament.

2.2 Behavior

The novel object recognition task was adapted from Leger and colleagues (Leger et al., 2013). This task consisted of 3 phases: In a first phase the animals freely explored the empty open-field arena for 10 min (habituation phase). Twenty four hours after, the animals were allowed to explore two similar, symmetrically disposed objects, for 10 min. Ten minutes before this phase the animals were submitted to intraperitoneal injection of either the drug or the vehicle, and stayed in an empty transport cage before entering the training phase. Six hours after the training phase, the animals were exposed to 2 objects located in the same positions as previously, but this time one of the objects was substituted by a new object that the animal had not contacted previously (test phase).

The object displacement test was adapted from Oliveira and colleagues (Oliveira et al., 2012). The test took place during 2 days. During the first day the animals were allowed to explore an empty open-field for 6 min (habituation phase). Immediately after, the animals were intraperitoneally injected with the inverse agonist of the ghrelin receptor or its respective vehicle and placed in their homecage. After 10 minutes, the animals explored two different objects placed in a specific location of the open field for 6 minutes (training phase). The animals were then returned to their homecage and waited for 3 minutes. Two more similar training phases were conducted, with a 3 minute waiting period in between. On the next day, the animals returned to the open field, where one of the objects was placed in a new location, and were allowed to explore the objects for 6 minutes (test phase).

The objects and their positions were randomized for both tests. The used objects correspond to the described by Leger and colleagues (Leger et al., 2013). The arena and the objects were carefully cleaned before running each animal and in between phases. The test was conducted at a room temperature of 23°C and 15 lux at the center of the arena (homogenously distributed light). Videos of the test were acquired using Noldus Ethovision software and scoring was performed blinded to treatment of the animals, using Noldus Observer.

Elevated plus maze was performed with a maze made in-house according to previously described specifications (Matsuo et al., 2010), and using an adapted protocol (2006). Animals were weighted and injected with the corresponding dose of inverse agonist or vehicle 10 min before starting the test and stayed in an empty transport cage. The test started by putting the animals in the central part of the maze with the nose aligned with the closed arms, and run for 10 min. The test was conducted under 100 lux at the center of the arena. The arena was carefully cleaned before and after each run. Videos of the test were acquired and automatically quantified using Noldus Ethovision.

2.3 DNA constructs

The GFP-tagged GHS-R1a construct (Leung et al., 2007) was a kind gift from Dr. Helen Wise (The Chinese University of Hong Kong) and SEP-GluA1 was generously provided from Helmut Kessels (Netherlands Institute for Neuroscience). For the generation of the short hairpin interfering RNA construct targeting the ghrelin receptor, a previously described and validated sequence (Shrestha et al., 2009) was used. Complementary oligonucleotides, each containing a unique 19-nt sequence derived from within the target mRNA transcripts of ghsr1a gene (NM_032075) targeting nucleotides 79-96 (GACTCACTGCCTGACGAAC) (Shrestha et al., 2009), were annealed and subcloned into the HpaI/XhoI sites of the U6 promoter-driven short hairpin RNA expression vector pLentiLox3.7(CMV)EGFP, which co-expresses EGFP under the CMV promoter. The control shRNA that targets firefly luciferase was described previously (Flavell et al., 2006). Homer1C-DsRed and Homer1C-GFP were previously described (Renner et al., 2009).

2.4 Materials

The GHS-R1a inverse agonist [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P (SP-A) was purchased from Bachem (Bubendorf, Switzerland), the AZ12861903 GHS-R1a inverse agonist was kindly provided by AstraZeneca. The GHS-R1a agonist MK-0677 was purchased from Axon Medchem (Groningen, The Netherlands). The GHS-R1a antagonist [D-Lys^{3}]-GHRP-6, TTX, and picrotoxin were purchased from Tocris Bioscience (Bristol, UK). The anti-Tubulin antibody was purchased from Sigma-Aldrich (Sintra, Portugal); the anti-Akt, anti-P-Ser473(Akt), anti-PSD95 (rabbit), and anti-CaMKIV antibodies were obtained from Cell Signaling (Danvers, MA, USA); the anti-GluA1, anti-GluA2, anti-P-Ser845(GluA1), anti-P-Ser239/240 (Stargazin) and anti-VGluT1 antibodies were from Millipore (Madrid, Spain); the anti-MAP2 antibody was from Abcam (Cambridge, UK), the anti-P-S831(GluA1) antibody was from Tocris Bioscience (Bristol,UK), the anti-PSD95 (mouse) antibody was from Affinity BioReagents (Golden, USA), and the anti-P-Thr196 (CaMKIV) antibody was from Santa Cruz Biotechnology Inc (Santa Cruz, CA); the anti-GFP (rabbit) antibody was from MBL International (Woburn, USA) and the anti-GFP (mouse) antibody was from Roche (Amadora, Portugal). Quantum dots (QDs) 655 Goat F(ab')2 anti-mouse IgG conjugate ($H^{+}L$) were purchased from Invitrogen (Barcelona, Spain). The antibody for the N-terminus of GluA1 was a kind gift from Dr. Andrew Irving (University College Dublin). All other reagents were purchased from Sigma-Aldrich (Sintra, Portugal), Fisher Chemicals or from Merck (Darmstadt, Germany) unless specified otherwise.

2.5 Application of GHS-R1a agonists and antagonists

Hippocampal organotypic slices (6 DIV) were treated with the GHS-R1a inverse agonist [D-Arg^{1}, D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P (SP-A, 1μM) for 20 h, or chronically treated with the GHS-R1a antagonist [D-Lys^{3}]-GHRP-6 (100 μM) from 3 DIV up to 7 DIV. Hippocampal neurons in culture were incubated with the GHS-R1a inverse agonists (SP-A and AZ12861903) and antagonist JMV2959. The compounds were added directly to the culture medium. AZ12861903 for injection in vivo was dissolved in 95% beta-hydroxypropylcyclodextrin (**β**-hpC)/5% v/v DMSO. **β**-hpC was prepared at 25 % w/v in Sorenson's buffer pH 5.5. All the injected solutions were prepared in sterile conditions. The drug and vehicle were injected intraperitoneally at volumes of 100-150 μl. The dose of 100 mg/kg was based on previously described doses by McCoull and colleagues (McCoull et al., 2014).

2.6 Neuronal and slice cultures

Primary cultures of rat hippocampal neurons were prepared as previously described (Santos et al., 2012). Hippocampal slices were prepared from young Wistar rats of either sex (postnatal day 5–6) as previously described (Gahwiler et al., 1997). DNA constructs [Luciferase shRNA-GFP, GHS-R1a shRNA-GFP (Knock-down), hGHS-R1a (rescue), SEP-GluA1, Homer1C-DsRed and Homer1C-GFP] were expressed in primary cultures of hippocampal neurons at 9 DIV using an adapted calcium phosphate transfection protocol (Jiang et al., 2004), as previously described (Santos et al., 2012).

2.7 Electrophysiology

Voltage-clamp whole-cell recordings were performed stimulating Schaffer collateral fibers and recording evoked synaptic responses from CA1 pyramidal neurons at different holding potentials. The AMPA/NMDA ratios were calculated by acquiring AMPA receptor responses at –60 mV and NMDA receptor responses at +40 mV at a latency at which AMPAR responses were fully decayed (60 ms after stimulation). Picrotoxin (100 μM) was present in the external solution to block the $GABA_A$ receptor responses. The recording chamber was perfused with external solution (in mM: 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 11 glucose, 26 $NaHCO_3$, 4 $MgCl_2$, 4 $CaCl_2$ and 0.004 2-chloroadenosine, at pH 7.4), and was gassed with 5% CO_2/ 95% O_2. Patch recording pipettes (3–6 M Ω) were filled with internal solution (in mM: 115 $CsMeSO_3$, 20 CsCl, 10 HEPES, 2.5 MgCl2, 4 Na_2ATP, 0.4 Na_3GTP, 10 sodium phosphocreatine and 0.6 EGTA, at pH 7.25). Synaptic responses were evoked with bipolar electrodes using single-voltage pulses (200 μs, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 300 and 500 μm from the CA1 recorded cells. Synaptic responses were averaged over 50 trials. Whole-cell recordings were carried out with a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, USA).

2.8 Immunocytochemistry

For labeling surface GluA1-containing AMPA receptors, live neurons were incubated for 10 min at room temperature using an antibody against an extracellular epitope in the GluA1 N-terminus diluted in conditioned neuronal culture medium or extracellular solution (used for chemical LTP). Neurons were then fixed and stained as previously described (Santos et al., 2012). For labeling GluA2-containing AMPA receptors, neurons were fixed and then incubated overnight with an anti-GluA2 antibody diluted 1:100 in 3%BSA/PBS, at 4°C. Neurons were then stained as previously described (Santos et al., 2012).

2.9 chemical LTP (cLTP) protocol

cLTP was induced as previously described (Ahmad et al., 2012). 19 DIV hippocampal cultures were washed with extracellular solution (ECS) containing (in mM): 150 NaCl, 2 $CaCl_2$, 5 KCl, 10 HEPES, 30 Glucose, 0.001 TTX, 0.01 strychnine, 0.03 picrotoxin, pH 7.4. After washing, neurons were stimulated with glycine (300 µM) at room temperature for 3 min in ECS and then incubated for 20–25 min in ECS in a 37°C, 5% CO_2/95% air incubator. Surface GluA1-AMPAR were labeled, and the cells were fixed and probed as described above.

2.10 Quantitative imaging analysis

Imaging was performed on a Zeiss Axio Observer Z1 microscope using a Plan Apochromat 63×/1.4 NA oil objective, and an AxioCam HRm CCD camera. Images were quantified using image analysis software (ImageJ). For quantification, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The region of interest was randomly selected avoiding primary dendrites, and dendritic length was measured using MAP2 staining. Measurements were performed in 2–5 independent preparations, and at least 7 cells per condition were analyzed for each preparation. Quantitative imaging quantification was performed as previously described (Santos et al., 2012).

2.11 Quantum dots labeling and Imaging

Endogenous GluA2 and GluA1-SEP labeling was performed in two steps: first neurons were incubated for 10 min at 37°C with anti-GFP antibody (1/300000) or anti-GluA2 antibody (1/1000), diluted in conditioned medium. After one washing step, anti-mouse IgG conjugated QD655 (diluted 1:10 in PBS) were diluted in conditioned medium with BSA 2% (1/2000) and were incubated on cells for 5 min at 37°C. Synapses were labeled using transfection with Homer1C-DsRed or Homer1C-GFP. All washes were performed in ECS containing (in mM) NaCl 145, KCl 5, Glucose 10, Hepes 10, $CaCl_2$ 2 and $MgCl_2$ 2), supplemented with BSA 2% at 37°C. After washing, neurons were mounted in an open chamber (K.F. Technology SRL) and imaged in ECS. Single-particle tracking was performed as in (Opazo et al., 2010). Cells were imaged at 37°C on an inverted microscope (AxioObserver Z1, Carl Zeiss) equipped with a Plan Apochromat 63× oil objective (NA = 1.4). QDs, Homer1C-DsRed and Homer1C-GFP signals were detected by using a HXP fluorescence lamp (For QDs: excitation filter 425/50 and emission filters 655/30, Chroma). Fluorescent images from QDs were obtained with an integration time of 50 ms with

up to 600 consecutive frames. Signals were recorded with a digital CMOS camera (ORCA Flash 4.0, Hamamatsu). QD-labeled GluAs were imaged on randomly selected dendritic regions over up to 30 min total experimental time. QDs fixed on the coverslip allowed us to compensate mechanical drifts of the stage.

2.12 Quantum dots Tracking and Analysis

The tracking of single QDs was performed with homemade software based on Matlab (Mathworks Inc., Natick, USA). Single QDs were identified by their diffraction limited signals and their blinking fluorescent emission. The trajectory of a QD tagged receptor could not be tracked continuously due to the random blinking events of the QDs. When the positions before and after the dark period were compatible with borders set for maximal position changes between consecutive frames and blinking rates, the subtrajectories of the same receptor were reconnected. The values were determined empirically: 2–3 pixels (0.32–0.48 μm) for maximal position change between two frames and maximal dark periods of 25 frames (1.25 s). MSD curves were calculated for reconnected trajectories of at least 20 frames. The QDs were considered synaptic if colocalized with Homer dendritic clusters for at least five frames. Diffusion coefficients were calculated by a linear fit of the first 4–8 points of the mean square displacement (MSD) plots versus time depending on the length of the trajectory within a certain compartment. The resolution limit for diffusion was 0.0075 $\mu m^2/s$ as determined by (Groc et al., 2004), whereas the resolution precision was ~40 nm. Statistical values are given as mean ± SEM or medians ± 25%/75% interval, if not stated otherwise. Statistical significances were performed using GraphPad Prism software. Non-Gaussian distributed data sets were tested by Mann-Whitney t-test. Indications of significance correspond to p values < 0.05 (*), p<0.01 (**), and p <0.001 (***).

2.13 Biochemistry

Protein extracts were prepared in lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100 supplemented with 1 mM DTT, 0.1 mM phenylmethylsulfonyl (PMSF), 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin (CLAP) and a cocktail of phosphatase inhibitors (1x, Roche, Carnaxide, Portugal)]. After centrifugation at 16,100 x g for 10 min at 4°C, protein in the supernatant was quantified using the bicinchoninic acid (BCA) assay kit (Pierce, Termo Fisher Scientific, Rockford, USA), and the samples were denatured with 5x concentrated denaturating buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) Glicerol, 2% (v/v) SDS, 0.01% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol (added fresh)], and boiled for 5 min. Protein extracts were resolved by SDS-PAGE in 7.5% or 12% polyacrylamide gels. For western blot analysis, proteins were transferred onto a PVDF membrane (Millipore, Madrid, Spain) by electroblotting (40 V, overnight at 4°C). The membranes were blocked for 1 h at room temperature in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% (v/v) Tween-20 (TBS-T), and 5% (w/v) low-fat milk or BSA. Membranes were probed during 1 h, at room temperature, or overnight, at 4°C, with the primary antibodies diluted in TBS-T containing 5% or 0.5% (w/v) low-fat milk or 5% (w/v) BSA. Following several washes, membranes were incubated for 1 h with alkaline phosphatase-conjugated secondary

antibodies (anti-mouse or anti- rabbit, depending on the primary antibody host species) at room temperature, washed again and incubated with chemifluorescent substrate (ECF) (GE Heathcare, Carnaxide, Portugal) for 5 min at room temperature. Membranes were scanned with the Storm 860 scanner (GE Heathcare, Carnaxide, Portugal), and quantified using the ImageQuant software under linear exposure conditions. When necessary, the membranes were stripped (0.2 M NaOH for 5 min) and re-probed.

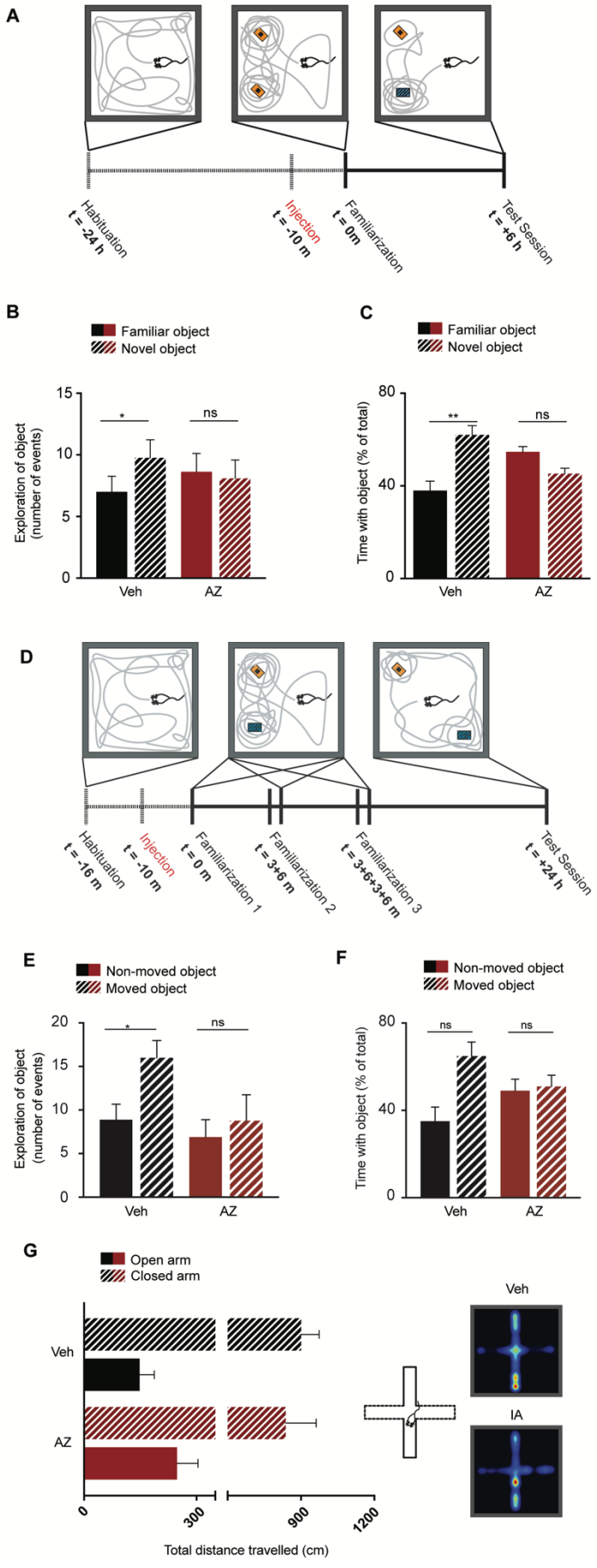
2.14 Statistical analysis

Statistical differences were calculated according to non-parametric tests in most cases; Mann-Whitney test was used to compare statistical differences between any two groups. Comparisons between multiple groups were performed with the Kruskal-Wallis analysis of variance followed by Dunn's multiple Comparison test or with 2 way ANOVA with Bonferroni test for correction of multiple comparisons.

3 Results

3.1 Constitutive activity of the ghrelin receptor is relevant for memory formation

To test whether ghrelin receptor constitutive activity plays a role in memory formation, we evaluated performance in the novel object recognition test (Leger et al., 2013) in mice injected with a newly described, blood-brain barrier (BBB) permeable inverse agonist of the ghrelin receptor, AZ12861903, which decreases the constitutive activity of the receptor (McCoull et al., 2014). During the familiarization session, animals were allowed to explore two identical objects for 10 min. After 6h, one of the objects was replaced with a novel object, and the percentage of time exploring either object was measured (test session, **Figure 3.1**). Whereas control animals explored the novel object a higher number of times and for longer, animals treated with the ghrelin receptor inverse agonist prior to the familiarization session did not show a preference for either object, as measured by the number of explorations of each object (**Figure 3.1 B**) or time spent with each object (**Figure 3.1 C**). To account for changes in motor behavior, before and after drug administration, we also measured total distance travelled by animals in the arena and did not find significant alterations between vehicle- and drug-treated animals (**Supplementary Figure 3.1A**).


A
Habituation
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Injection
t = -10 m
Familiarization
t = 0m
Test Session
t = +6 h
B
Familiar object
Novel object
Exploration of object (number of events)
15
10
5
0
*
ns
Veh
AZ
C
Familiar object
Novel object
Time with object (% of total)
80
40
0
**
ns
Veh
AZ
D
Habituation
t = -16 m
Injection
t = -10 m
Familiarization 1
t = 0 m
Familiarization 2
t = 3+6 m
Familiarization 3
t = 3+6+3+6 m
Test Session
t = +24 h
E
Non-moved object
Moved object
Exploration of object (number of events)
20
15
10
5
0
*
ns
Veh
AZ
F
Non-moved object
Moved object
Time with object (% of total)
80
40
0
ns
ns
Veh
AZ
G
Open arm
Closed arm
Veh
AZ
0
300
900
1200
Total distance travelled (cm)
Veh
IA

Figure 3.1 - Administration of an inverse agonist of the ghrelin receptor impairs recognition and spatial memory.
(A–F) Male C57/BL6 mice were divided into two groups, vehicle (Veh)-treated animals and animals receiving 100 mg/kg of AZ12861903 (AZ). (A-C) Mice were habituated to the arena for 10 min without any objects and, after a delay of 24 h, were injected i.p. with either vehicle or 100 mg/kg AZ, before being re-introduced for 10 min in the arena now containing two identical objects. Following a delay of 6 h, animals were re-introduced in the arena where a novel object replaced one of the familiar objects, and animals explored the arena for 10 min. Only the first 5 min of the test were considered. (A) Schematic representation of a novel object recognition test with "habituation", "drug injection", "familiarization" and "test session" time line. (B) Number of interactions with objects during test session is expressed as a percentage (± SEM) of all interactions with objects, for vehicle- and AZ-treated groups; n=13 Veh, n=11 AZ. (C) Time spent with objects during the test session is expressed as a percentage (± SEM) of total duration of interaction with both objects, for vehicle- and AZ-treated groups. (D-F) A new cohort of mice were habituated to the arena for 6 min in the absence of objects and were injected with either vehicle or 100 mg/kg AZ immediately after. Injected animals were returned to the homecage for 10 min before returning to the arena where two different objects were placed in a specific location. Mice were allowed to explore the objects for 6 mins (familiarization), and this session was repeated 3 times with 3 min interval between each repetition. After 24 h, the animals were returned to the arena, where one of the objects was placed in a new location, and were allowed to explore for 6 min. (D) Schematic representation of an object displacement recognition test with "habituation", "drug injection", "familiarization" and "test session" time line. (E) Number of interactions with objects during test session is expressed as a percentage (± SEM) of all interactions with objects, for vehicle- and AZ-treated groups; n=8 Veh, n=9 AZ. (F) Time spent with objects during the test session is expressed as a percentage (± SEM) of total duration of interaction with both objects, for vehicle- and AZ-treated groups. (B,C,E,F) Comparisons between groups were performed using the Wilcoxon matched-pairs signed rank test; * $P<0.05$, ** $P < 0.01$. (g) Total distance travelled in an elevated plus maze was assessed to determine anxiety-like behavior following administration of AZ at 100 mg/kg (n=5) when compared to vehicle injections (n=6). No significant differences were found in distance travelled in the open arms of the elevated plus maze, Mann-Whitney test $P > 0.05$. See also Figure S1.

To test whether the constitutive activity of the ghrelin receptor is relevant for spatial memory, we used the object displacement test (Oliveira et al., 2012) (**Figure 3.1 D**). During the habituation session the animals explored an open-field arena in the absence of objects for 6 min; immediately after, the animals were treated with ghrelin receptor inverse agonist or vehicle injection. The animals returned to the open-field 10 min post-injection, and 2 different objects were present in specific locations. This familiarization session lasted 6 min and was repeated twice. After 24h, the animals were tested in the open-field with one of the objects displaced to a different location. Our results show that animals injected with vehicle preferentially explored the moved object, whereas animals injected with the inverse agonist did not show such preference (**Figure 3.1 E-F**). Total distance travelled was not affected by injection of the ghrelin receptor agonist (**Supplementary Figure 3.1B**). Since anxiolytic effects have been observed in mice administered with ghrelin (Lutter et al., 2008), we tested performance in the elevated plus maze after blocking constitutive activity of the ghrelin receptor. Consistent with prior results using knock-out mice for the ghrelin receptor (Lutter et al., 2008), blockade of the constitutive activity of the ghrelin receptor did not affect performance in the elevated plus maze (**Figure 3.1 G, Supplementary Figs. 3.1C,D**).

Our results indicate that acute blockade of GHSR-1a constitutive activity impairs performance during the novel object recognition task and in the object displacement recognition task, which suggests that tonic activity of the ghrelin receptor is important for learning and memory.

3.2 AMPA-receptor surface and synaptic expression is regulated by ligand-independent activity of the ghrelin receptor

In order to understand the molecular mechanism through which the constitutive activity of the ghrelin receptor may impact memory formation, we tested whether inverse agonists of the ghrelin receptor affect the synaptic content of AMPA-type glutamate receptors in cultured hippocampal neurons, given the role of the hippocampus and excitatory transmission in spatial memory. We used the AZ12861903 (AZ) compound (McCoull et al., 2014) and also the more widely used [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (SP-A), a well-established ghrelin receptor inverse agonist which does not cross the BBB (Holst et al., 2003a). Incubation of hippocampal neurons (15 DIV) with either of these compounds decreased the cell surface total levels of GluA1 (**Supplementary Figs. 3.2A,** B), and the levels of cell surface GluA1-containing AMPA receptors colocalized with the postsynaptic protein PSD95 and the presynaptic protein VGluT1 (**Figure 3.2 A-D**). In contrast, incubation with the ghrelin receptor antagonist JMV2959 did no significantly affect the total or synaptic levels of surface GluA1 (**Supplementary Figs. 3.2C,D**). Similarly to GluA1, GluA2 synaptic levels were decreased in neurons incubated with SP-A (**Supplementary Figs. 3.2E,F**). However, the incubation with SP-A did not affect synapse density in 15 DIV cultured hippocampal neurons (**Supplementary Figure 3.2G**), measured by the colocalization of PSD95 and VGluT1 puncta. SP-A also decreased the total surface and synaptic levels of GluA1, as well as synapse density in older neurons (20 DIV, **Supplementary Figs. 3.2H-J**), but not in 7 DIV neurons (**Supplementary Figs. 3.2K-M**), which at this age present lower levels of expression of the ghrelin receptor (Ribeiro et al., 2014).

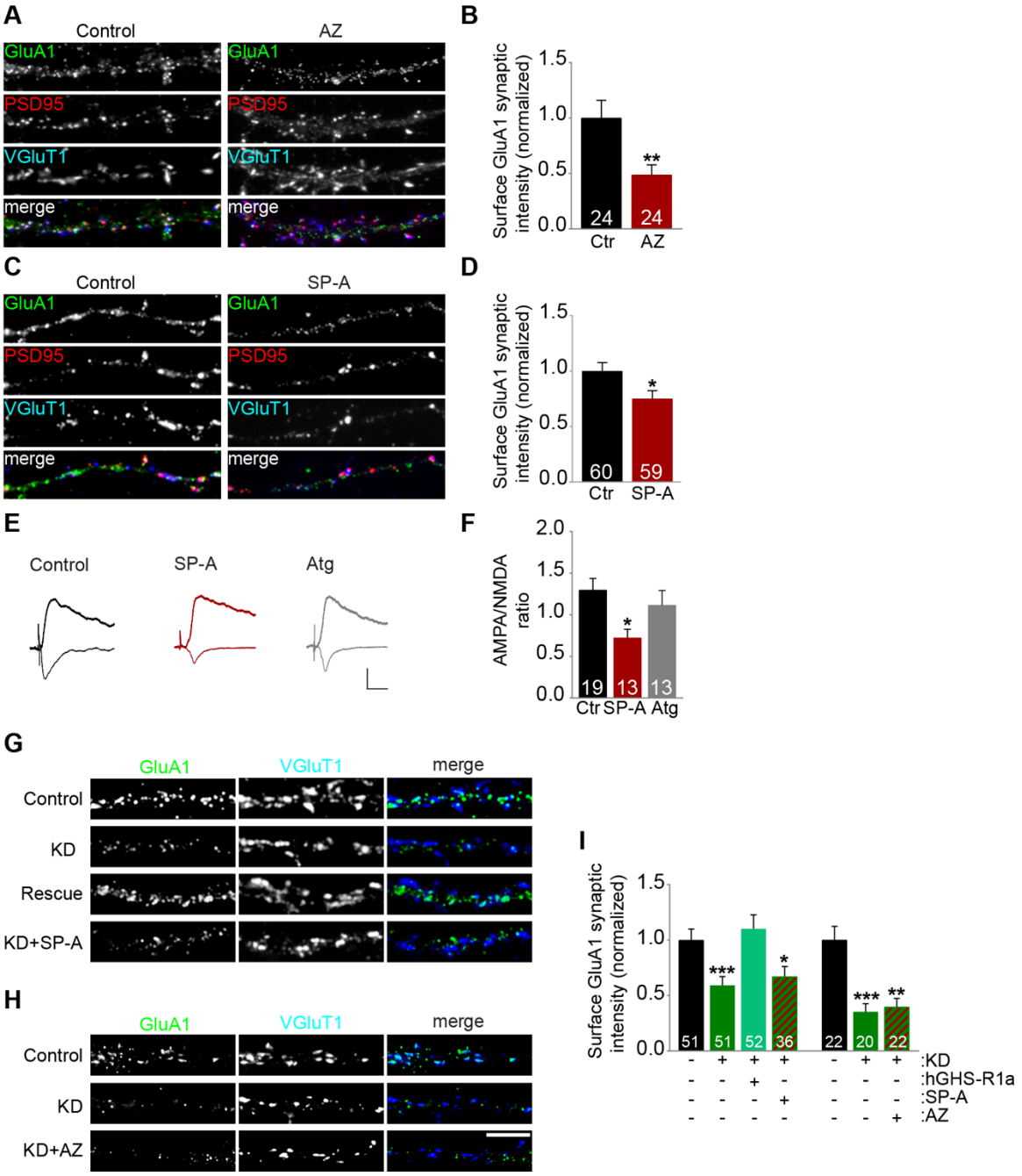


Figure 3.2 - Ligand-independent activity of the ghrelin receptor controls synaptic levels of AMPAR and excitatory synaptic transmission in the hippocampus.
(A,C) Hippocampal neurons [15 d in vitro (DIV)] were incubated with ghrelin receptor inverse agonists AZ12861903 (AZ) 50 nM (A) or [D-Arg1,D-Phe5,D-Trp7,9,Leu11]Substance P (SP-A) 1 μM (C) for 20 h and immunostained for surface GluA1 under non-permeabilizing conditions. After permeabilization, neurons were stained for MAP2, PSD95 and VGluT1 and analyzed for the total fluorescence intensity of GluA1 synaptic cluster (VGluT1/PSD95-colocalized) per synapse density. (B,D) Results are expressed as the percentage of control cells, and are averaged from 2–4 independent experiments. *P < 0.05 and **P < 0.01 by Mann-Whitney test. (E) Comparison of evoked synaptic AMPAR and NMDAR currents in 7 DIV organotypic hippocampal slices, in control condition, upon treatment for 20 h with SP-A, or with the ghrelin receptor antagonist (Atg; [D-Lys3]-GHRP-6, 100 μM). Scale bars: vertical, 50 pA; horizontal, 20 ms. (f) Average AMPA/NMDA ratios for SP-A-, antagonist-treated and control cells. *P < 0.05 by Kruskal-Wallis test followed by Dunn's post hoc test. (G, H) 15 DIV hippocampal neurons were transfected with constructs encoding luciferase shRNA-GFP (control), GHS-R1a shRNA-GFP (KD),

GHS-R1a shRNA-GFP + hGHS-R1a (rescue) or GHS-R1a shRNA-GFP. Neurons expressing GHS-R1a shRNA-GFP were treated with SP-A (G) or AZ (H) for 20h. Neurons were immunostained for surface GluA1 under non-permeabilizing conditions. After permeabilization, neurons were stained for GFP, MAP2 and VGluT1, and analyzed for the total fluorescence intensity of GluA1 synaptic cluster (VGluT1-colocalized) per density of VGluT1 clusters. Scale bar represents 5 μm. (I) Results are expressed as the percentage of control cells, and are averaged from 3–4 independent experiments. *P < 0.05 and ***P < 0.001 by Kruskal-Wallis test followed by Dunn's post hoc test. All data are presented as mean ± SEM, and number in bars indicates number of cells analyzed for each condition. See also Figure S2.

To test whether the ligand-independent activity of the ghrelin receptor functionally modulates CA3-CA1 hippocampal excitatory transmission, organotypic hippocampal slices were treated with SP-A for 20h, and electrophysiological recordings were performed. The AMPA/NMDA ratio of synaptic responses decreased significantly after treatment with SP-A, compared with control neurons, whereas the ghrelin receptor antagonist [D-Lys3]-GHRP-6 did not affect AMPA/NMDA ratios (**Figure 3.2** E, F), in agreement with low levels of ghrelin in the culture, and a specific role for the ghrelin receptor inverse agonist in inhibiting CA3-CA1 synaptic transmission. Together, these results suggest that the constitutive activity of GHSR regulates AMPA receptor levels and excitatory synaptic transmission under basal conditions.

We then silenced the expression of the ghrelin receptor in cultured hippocampal neurons using interference RNA (shRNA) and tested for surface levels of GluA1. Knock-down of the ghrelin receptor decreased the levels of surface and synaptic GluA1; this effect was rescued when the shRNA was co-expressed with an shRNA-insensitive human form of the receptor (hGHS-R1a, **Figure 3.2** G-I, **Supplementary Figure 3.2N**). Notably, treatment with either SP-A or AZ did not affect the total surface levels or synaptic levels of GluA1 in neurons where the ghrelin receptor expression was silenced (**Figure 3.2 G-I**), confirming the specificity of SP-A and AZ in targeting the ghrelin receptor.

Since the constitutive activity of the ghrelin receptor promotes its own basal internalization, we reasoned that ligand-independent activity could limit ghrelin-induced effects in the hippocampus (Ribeiro et al., 2014). To test this, we used 15 DIV cultured hippocampal neurons, for which no effect of ghrelin receptor activation on AMPA receptor surface expression was detected (**Supplementary Figs. 3.3A-C**). We found that upon blockade of ghrelin receptor constitutive activity with SP-A, subsequent activation of the ghrelin receptor with the agonist MK677 increased the surface and synaptic expression of AMPA receptors (**Supplementary Figs. 3.3A-C**). These data indicate that the hippocampal ghrelin receptor ligand-independent activity promotes tonic expression of synaptic AMPA receptors (**Figure 3.2**), and on the other hand limits the effects of ghrelin on AMPA receptor traffic (**Supplementary Figs. 3.3A-C**).

3.3 Ligand-independent activity regulates AMPA receptor surface mobility

To determine how the ligand-independent activity of the ghrelin receptor may regulate AMPA receptors, we tested for effects on the cell surface diffusion of GluA1 AMPA receptor subunit. We expressed superecliptic pHluorin (SEP)-GluA1 in cultured hippocampal neurons and took advantage of the single particle tracking approach to monitor individual AMPA receptor complexes (**Figure 3.3 A-E**). Hippocampal neurons were exposed to SP-A for 1 h and single nanoparticle imaging of SEP-GluA1 was performed thereafter. SP-A exposure significantly increased the surface diffusion of GluA1 (both the mean square displacement and diffusion coefficient were increased **Figure 3.3 A,B**), decreased the fraction of synaptic immobile receptors (**Figure 3.3 C**) and decreased the synaptic residence time of GluA1-AMPA receptors (**Figure 3.3 D**).

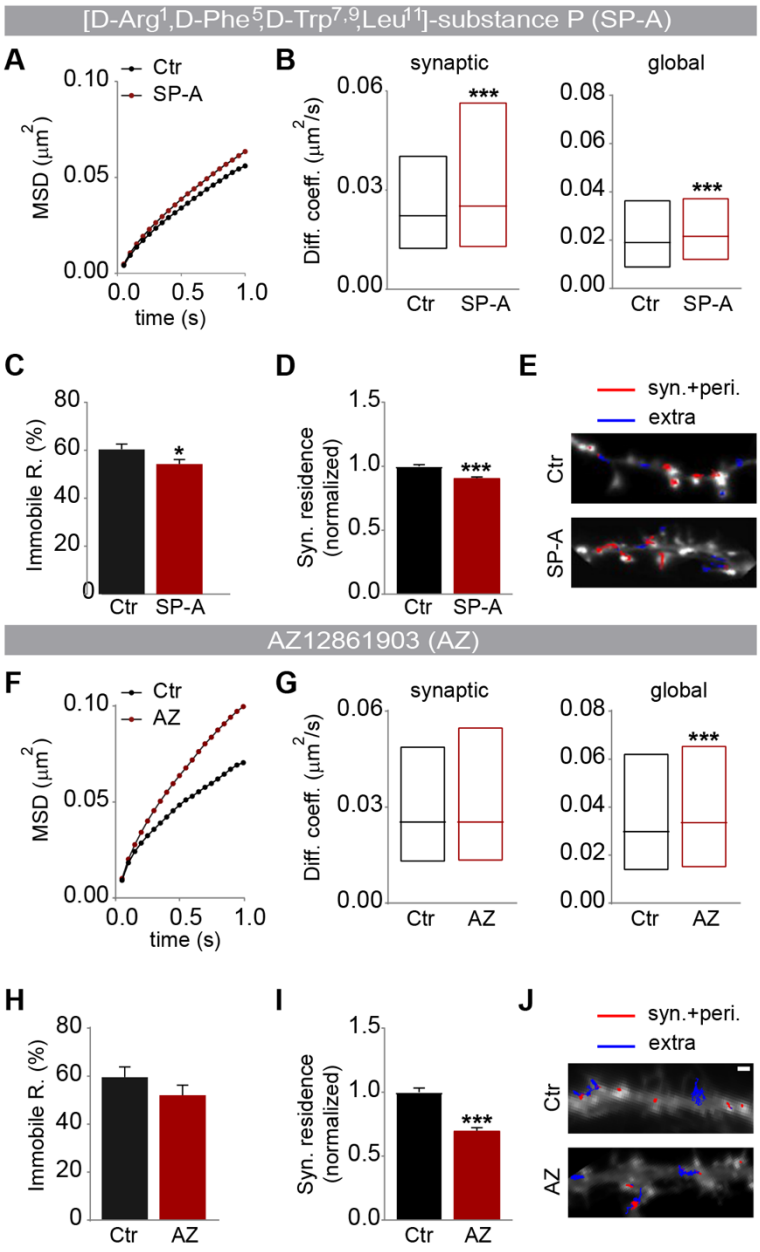


Figure 3.3 - Blockage of the ligand-independent activity of the ghrelin receptor increases the cell surface diffusion of AMPAR.

(A–E) Hippocampal neurons co-transfected with SEP-GluA1 and Homer1C-DsRed (11DIV) were incubated with SP-A 1 μM for 1 h (15DIV) prior to assessing GluA1 surface diffusion using quantum dots labelled antibodies for GFP to detect SEP-GluA1 (QD-GluA1). (A) GluA1 mean square displacement (MSD) versus time plots for control and SP-A-treated cells. (B) Surface diffusion coefficient of synaptic (left) and global (right) single QD-GluA1. Median diffusion (± 25%–75% IQR) of 8816–8607 trajectories. ***P < 0.001 by Mann-Whitney test. (C) Mean percentage (± SEM) of synaptic immobile GluA1-AMPAR in control and SP-A-treated cells. *P < 0.05 by Mann-Whitney test. (D) Mean synaptic residence time (± SEM) of GluA1-AMPAR in control and SP-A-treated cells. ***P < 0.001 by Mann-Whitney test. (E) Reconstructed GluA1 trajectories in the synaptic (red) and extrasynaptic compartments (blue). A minimum of 37 cells were analyzed in 3 independent experiments. (F–J) Hippocampal neurons transfected with Homer1C-DsRed (11 DIV) were incubated with AZ 50 nM for 1 h (15 DIV) prior to assessing GluA1 surface diffusion using quantum dots-labelled antibodies for GluA1 (QD-GluA1). (F) GluA1 mean square displacement (MSD) versus time plots for control and AZ-treated cells. (G) Surface diffusion coefficient of synaptic (left) and global (right) single QD-GluA1. Median diffusion (± 25%–75% IQR) of 1770–1303 trajectories. ***P < 0.001 by Mann-Whitney test. (H) Mean

percentage (± SEM) of synaptic immobile GluA1-AMPAR in control and AZ-treated cells. (I) Mean synaptic residence time (± SEM) of GluA1-AMPAR in control and AZ-treated cells. ***P < 0.001 by Mann-Whitney test. (J) Reconstructed GluA1 trajectories in the synaptic (red) and extrasynaptic compartments (blue). 41 cells per condition were analyzed in 3 independent experiments. Scale bar represents 1 µm. See also **Supplementary Figure 3.4**.

We repeated this experiment now applying AZ and using quantum-dots labeled antibodies against an extracellular region in GluA1, to follow endogenous AMPA receptors (**Figure 3.3 F-J**). Endogenous GluA1 also showed increased mean square displacement and diffusion coefficient and decreased synaptic residence time in neurons treated with AZ. These data provide evidence that the constitutive activity of the ghrelin receptor contributes to decrease the surface diffusion of synaptic AMPA receptors, thereby increasing the synaptic content of AMPA receptors under basal conditions in hippocampal neurons.

3.4 Effects of the constitutive activity of the ghrelin receptor on activity-induced synaptic incorporation of AMPA-receptor

We then assessed whether activity-induced synaptic incorporation of AMPA receptors is regulated by the ligand-independent activity of the ghrelin receptor. We used a neuronal culture model of chemical long-term potentiation (cLTP), in which activation of NMDA receptors triggers an increase in the expression of surface synaptic AMPA receptors (Ahmad et al., 2012). In agreement with previous reports, application of glycine, the co-agonist of NMDA receptors, in the absence of Mg2+ led to a significant increase in the synaptic expression of GluA1-AMPA receptors compared to control cells (**Figure 3.4 A-D**). However, this effect was blocked in neurons pre-incubated with either SP-A (**Figure 3.4 A, B**) or AZ (**Figure 3.4 C, D**), indicating that GHSR constitutive activity is necessary for AMPA receptors synaptic insertion upon cLTP.

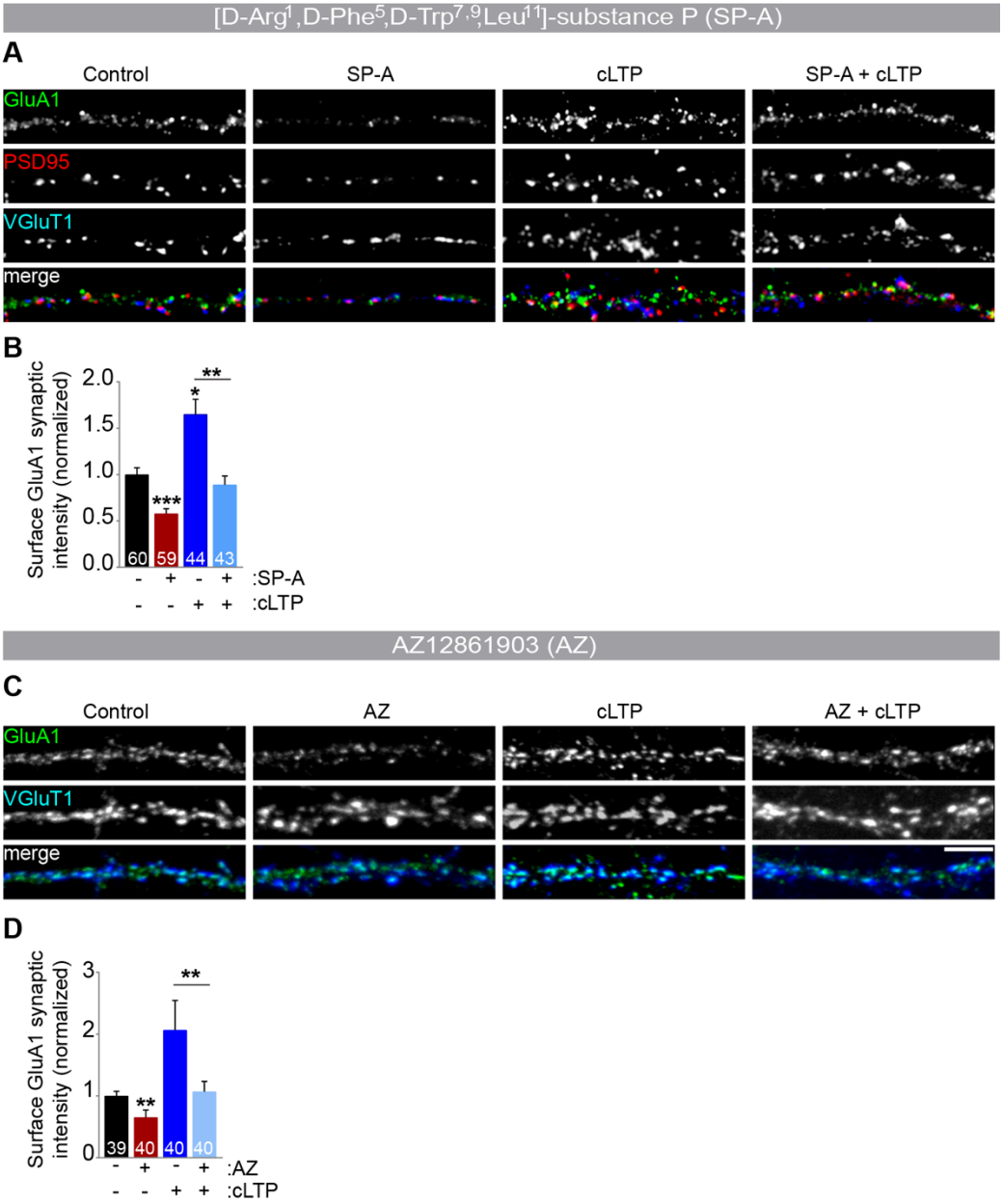


Figure 3.4 - Blockage of the ligand-independent activity of the ghrelin receptor prevents the activity-triggered delivery of AMPAR to synaptic sites.
(A) cLTP-induced insertion of synaptic GluA1. 19–20 DIV hippocampal neurons were either treated with SP-A for 20 h, submitted to cLTP, or pre-treated with SP-A and submitted to cLTP. Neurons were immunostained for surface GluA1 under non-permeabilizing conditions and analyzed for total fluorescence intensity of GluA1 synaptic clusters (VGLUT1/PSD95-colocalized) per synapse density. (B) Results are expressed as the mean percentage of control cells (± SEM), and are averaged from 3–4 independent experiments. Number in bars indicates number of cells analyzed for each condition. *P < 0.05, **P < 0.01 and ***P < 0.001 by Kruskal-Wallis test followed by Dunn's post hoc test. (C) 19–20 DIV hippocampal neurons were either treated with AZ for 20 h, submitted to cLTP, or pre-treated with AZ and submitted to cLTP. Neurons were immunostained for surface GluA1 under non-permeabilizing conditions and analyzed for total fluorescence intensity of GluA1 synaptic clusters (VGluT1-colocalized) per synapse density. Scale bar represents 5 µm. (D) Results are expressed as the mean percentage of control cells (± SEM), and are averaged from 3–4 independent experiments. Number in bars indicates number of cells analyzed for each condition. **P < 0.01 by Kruskal-Wallis test followed by Dunn's post hoc test.

3.5 Intracellular signaling of ligand-independent activity of the ghrelin receptor

We finally tested cell signaling pathways downstream of the ghrelin receptor constitutive activity that could result in altered trafficking of AMPA receptors. We found that upon blockade of the ligand-independent activity of the ghrelin receptors in organotypic hippocampal slices there was a decrease in the phosphorylation of GluA1 at Ser845 (**Figure 3.5 A**), a PKA phosphorylation site critical for priming AMPA receptors for synaptic insertion (Holliday et al., 2007). Additionally, a decrease in the phosphorylation of CaMKIV (**Figure 3.5 B**) was detected, whereas no changes were found in the phosphorylation of GluA1 at Ser831, in the phosphorylation of stargazin or in the phosphorylation of Akt (**Figure 3.5 C-E**). These results suggest that PKA activation downstream of the constitutive activity of the ghrelin receptor may result in phosphorylation of GluA1 at Ser845 and contribute to maintaining a population of AMPA receptors available for synaptic insertion.

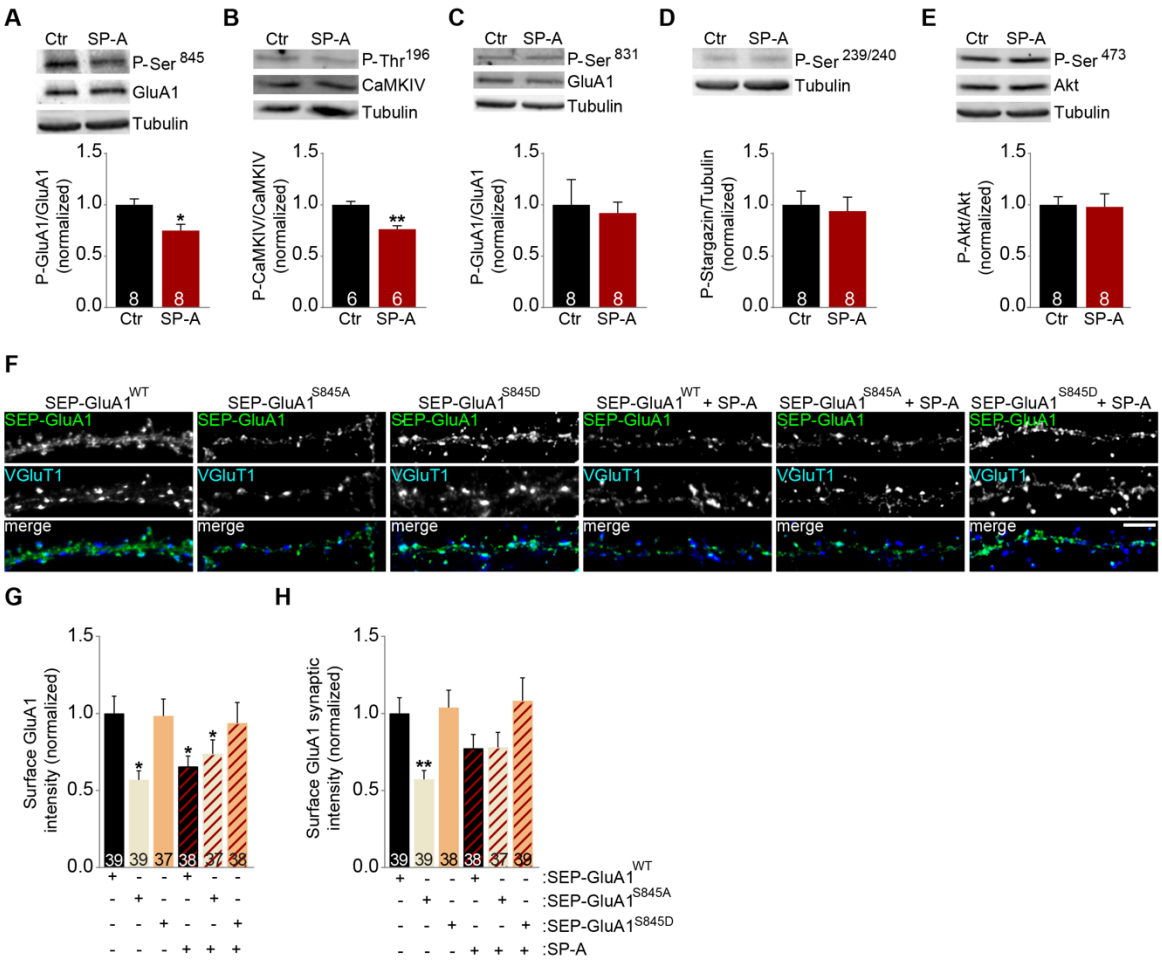


Figure 3.5 - Signaling pathways downstream of the ghrelin receptor constitutive activity.
(A-E) Western blot analysis of protein extracts from 7 DIV organotypic hippocampal slices incubated with culture medium or medium containing SP-A (1 μM) for 20 h. Primary antibodies detected

phosphorylation of GluA1 at Ser845 (A), phosphorylarion of CaMKIV at Thr196 (B), phosphorylation of GluA1 at Ser831 (C), phosphorylation of stargazin at Ser239/240 (D), phosphorylarion of Akt at Ser473 (E), a residue targeted by a PI3 kinase downstream signaling pathway. Total GluA1 (A, C), total CaMKIV (B) and total Akt (E) were also detected. Tubulin was used as a loading control in all cases. The graphs represent the quantification of band intensities relative to control extracts. Error bars represent SEM. The statistical significance was calculated using the Mann-Whitney test (*P < 0.05 and **P < 0.01). n represents the number of independent experiments. (F-H) 15 DIV hippocampal neurons expressing SEP-GluA1 (control), SEP-GluA1S845A or SEP-GluA1S845D, non-treated or treated with SP-A for 20 h, were immunostained for surface GFP under non-permeabilizing conditions. After permeabilization, neurons were labeled with Alexa568-conjugated secondary antibody to reveal surface GluA1. Neurons were also stained for MAP2 and VGluT1, and analyzed for the cell-surface fluorescence intensity of GFP clusters, total and synaptic (VGluT1-colocalized), per density of VGluT1 clusters. Scale bar represents 5 µm. (G,H) Results are expressed as the percentage of control cells, and are averaged from 3 independent experiments. *P < 0.05 and **P < 0.01 by Kruskal-Wallis test followed by Dunn's post hoc test. All data are presented as mean ± SEM, and number in bars indicates number of cells analyzed for each condition.

To test whether the ghrelin receptor constitutive activity contributes to AMPA receptor traffic through effects on GluA1 phosphorylation at Ser845, we evaluated whether SP-A affects the cell surface and synaptic levels of phosphodead and phosphomimetic mutants of GluA1 at Ser 845 (Ser845A and Ser845D, respectively). We found that contrarily to wild-type GluA1, SP-A treatment did not alter the cell surface or synaptic levels of either of these mutants (**Figure 3.5 F-H**), suggesting GluA1 phosphorylation at Ser845 as one mechanism through which the constitutive activity of the ghrelin receptor regulates AMPA receptor traffic.

3.6 Effects of the direct administration of ghrelin receptor inverse agonist to the hippocampus using intracerebroventricular injections

To test the direct effects of the constitutive activity of the receptor in the hippocampus we implanted cannulas (Bilaney Consultants Gmbh, Germany) bilaterally in the dorsal hippocampus in the border between CA1 and the dentate gyrus (rostrocaudal: 1.8 mm, lateral: 1 mm, dorsoventral: 1.6 mm). Implantation surgery was performed under anesthesia (isofluorane) and animals were administered with buprenorphine and meloxicam before each procedure. Minocycline was given to the animals during the first 24h of recovery. After this recovery period, animals were given a dose of buprenorphine and meloxicam and housed individually until behavioral testing.

Behavior tests were conducted in a similar manner as when drugs were administered intraperitoneally. The procedure differed in that in this case, before each training behavior run, animals were anesthetized and administered a 0.5 µL of either AZ (33.6 mM) or Vehicle. The vehicle used was similar to the one used for intraperitoneal injections. Injections were given at speed of 0.16 µL/min and the internal fit was maintained inside the cannula for additional 1 minutes in order to allow the drug to disperse. Animals were left to recover from anesthesia for 15 minutes, until normal condition was verified, and the test was run. Cannula position was verified with muscimol administration and running of object displacement

recognition test (memory was impaired in injected animals), and with injection of violet cresyl and subsequent histological processing.

We did not observe significant differences in learning and memory of animals injected with the inverse agonist directly into the hippocampus, as evaluated by the object displacement and the novel object recognition task (**Figure 3.6**). We also did not observe significant differences in the performance of injected animals in a contextual fear memory test (**Figure 3.7**), although we observed a slight tendency towards a decrease in the acquisition/expression of this type of memory in animals injected with the inverse agonist.

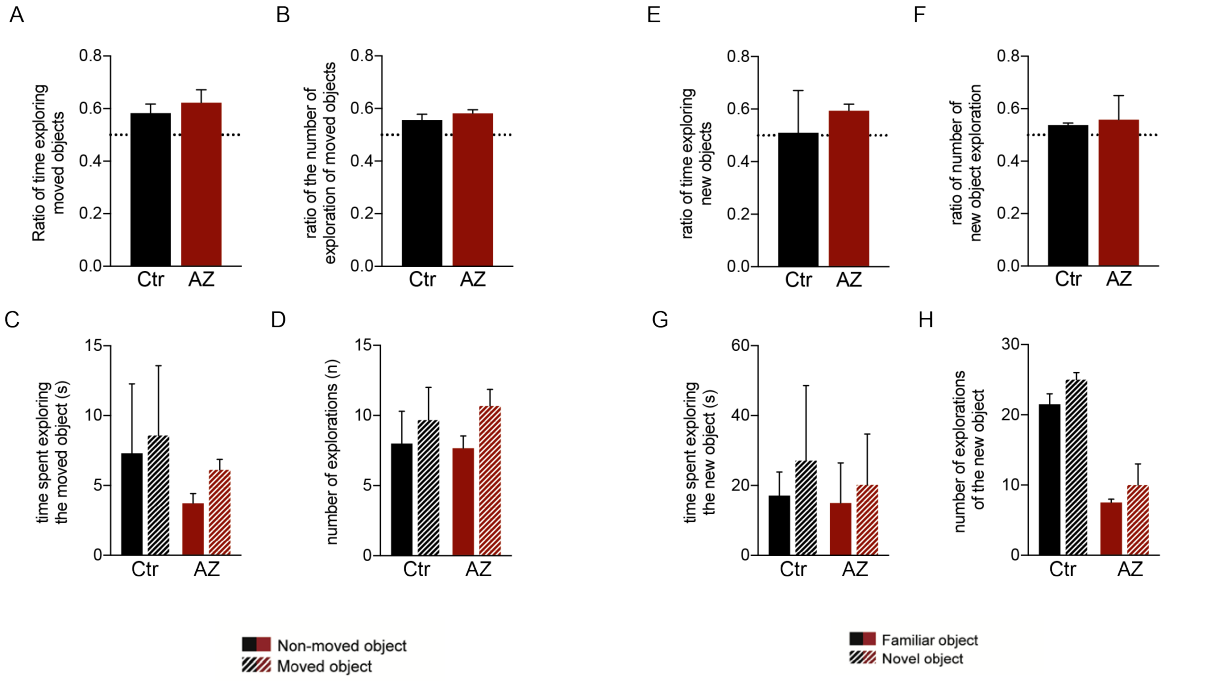


Figure 3.6 – Intrahippocampal injections of AZ inverse agonist do not affect object displacement (A-D) and novel object recognition memory (E-F). A) Ratio of time spent exploring the moved objects Ctr n=3, AZ n=3; B) Ratio of the number of times the animals explored the moved objects Ctr n=3, AZ n=3; C) Total time spent exploring each object Ctr n=3, AZ n=3; D) Total number of explorations of each object Ctr n=3, AZ n=3; E) ratio of time exploring the novel objects Ctr n=2, AZ n=2; F) ratio of number of novel object explorations Ctr n=2, AZ n=2; G) total time spent exploring the new object Ctr n=2, AZ n=2; H) total number of explorations of the new objects Ctr n=2, AZ n=2. No statistical significant differences were found in any of the measures.

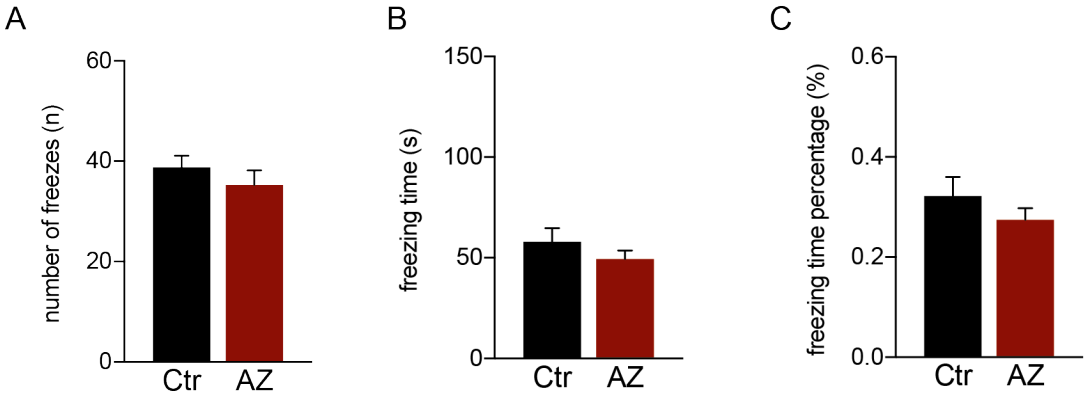
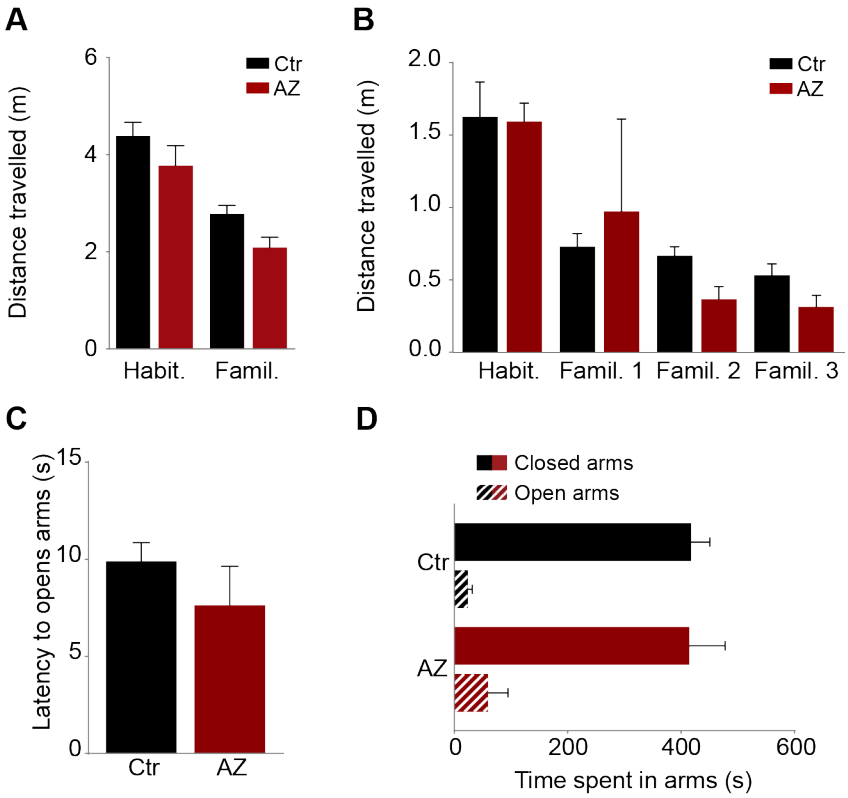


Figure 3.7 - Intrahippocampal injections of AZ inverse agonist of the ghrelin receptor effect on contextual fear memory. A) number of freezes animals performed during the test session. B) Sum of freezing time for each animal over the test session. C) percentage of the total duration of the test session spent freezed. No statistically significant differences were found in any of the measures. Ctr = 9, AZ = 8 No statistical significant differences were found in any of the measures

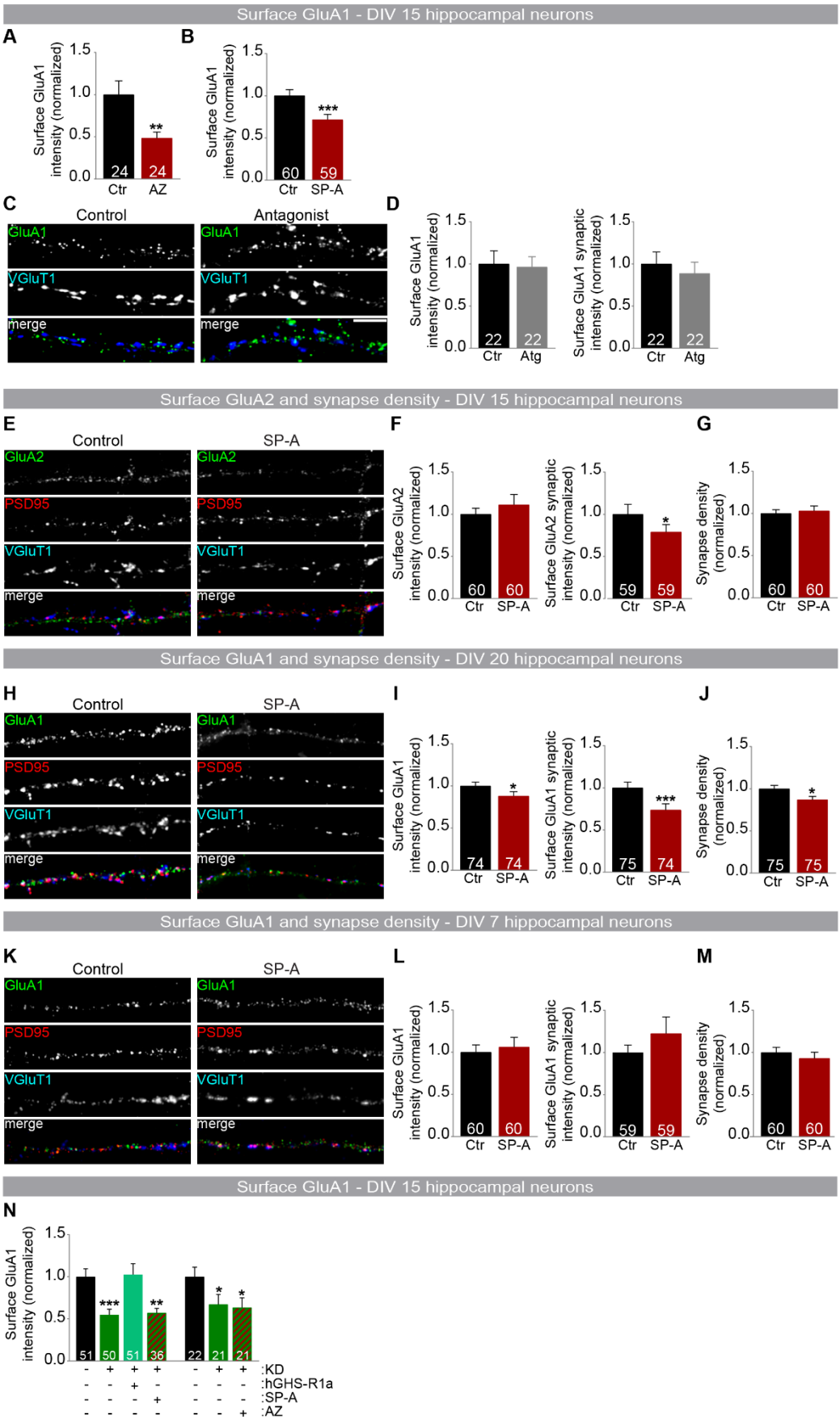
4 Supplementary Results

4.1 Ghrelin receptor constitutive activity is not involved in anxiety and does not control animal motility



Supplementary Figure S 3.1 (Related to Figure 3.1) Blocking the constitutive activity of the ghrelin receptor did not affect the motility of animals
(**A**) Distance travelled (± SEM) by each animal during the whole duration of novel object recognition test sessions: "habituation" and "familiarization"; n=13 vehicle (Ctr), n=11 AZ12861903. (**B**) Distance travelled (± SEM) by each animal during the whole session of the object displacement recognition test sessions: "habituation" and "familiarization"; n=8 vehicle (Ctr), n=9 AZ12861903. Comparison between groups was performed using 2 way ANOVA with Bonferroni test for correction of multiple comparisons. (**C**) Latency to enter open arms (± SEM) and (**D**) time spent on each arm of the elevated plus maze (± SEM); n=6 Ctr, n=5 AZ. No significant differences were found for either measurements between groups, using Mann-Whitney test.

4.2 Ghrelin receptor constitutive activity regulates synaptic levels of GluA2, the regulation of GluA1 levels shows a dependence on the age of the cultures and the phenotype of the exposure to ghrelin receptor inverse agonist is similar to that of the knockdown of the ghrelin receptor

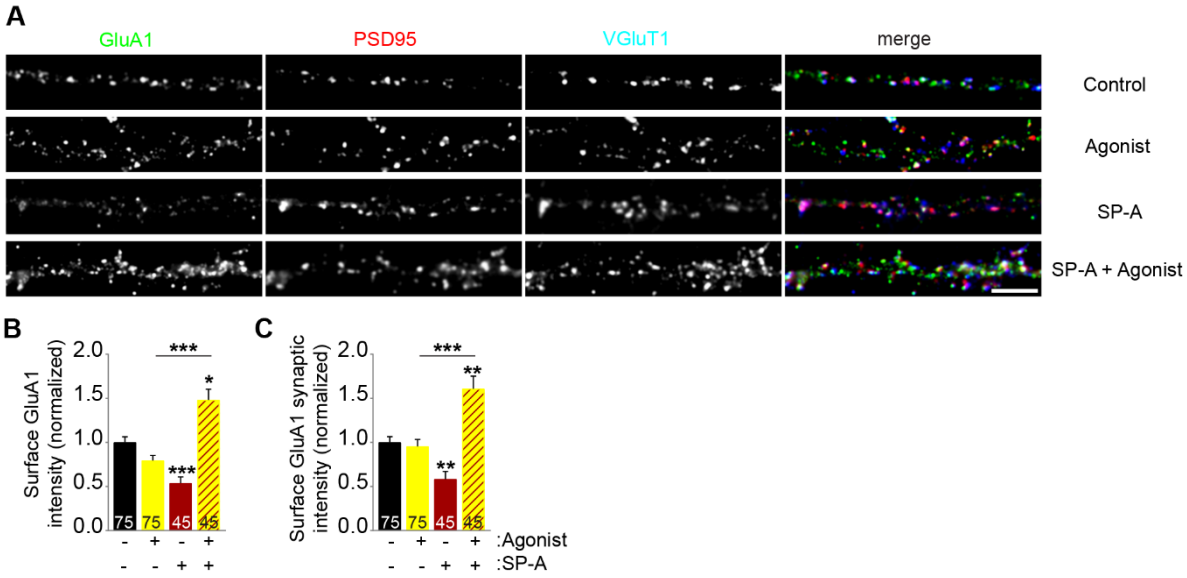

Surface GluA1 - DIV 15 hippocampal neurons
A
Surface GluA1 intensity (normalized)
1.5
1.0
0.5
0.0
**
24
24
Ctr
AZ
B
Surface GluA1 intensity (normalized)
1.5
1.0
0.5
0.0
***
60
59
Ctr
SP-A
C
Control
Antagonist
GluA1
VGluT1
merge
D
Surface GluA1 intensity (normalized)
1.5
1.0
0.5
0.0
22
22
Ctr
Atg
Surface GluA1 synaptic intensity (normalized)
1.5
1.0
0.5
0.0
22
22
Ctr
Atg
Surface GluA2 and synapse density - DIV 15 hippocampal neurons
E
Control
SP-A
GluA2
PSD95
VGluT1
merge
F
Surface GluA2 intensity (normalized)
1.5
1.0
0.5
0.0
60
60
Ctr
SP-A
Surface GluA2 synaptic intensity (normalized)
1.5
1.0
0.5
0.0
*
59
59
Ctr
SP-A
G
Synapse density (normalized)
1.5
1.0
0.5
0.0
60
60
Ctr
SP-A
Surface GluA1 and synapse density - DIV 20 hippocampal neurons
H
Control
SP-A
GluA1
PSD95
VGluT1
merge
I
Surface GluA1 intensity (normalized)
1.5
1.0
0.5
0.0
*
74
74
Ctr
SP-A
Surface GluA1 synaptic intensity (normalized)
1.5
1.0
0.5
0.0
***
75
74
Ctr
SP-A
J
Synapse density (normalized)
1.5
1.0
0.5
0.0
*
75
75
Ctr
SP-A
Surface GluA1 and synapse density - DIV 7 hippocampal neurons
K
Control
SP-A
GluA1
PSD95
VGluT1
merge
L
Surface GluA1 intensity (normalized)
1.5
1.0
0.5
0.0
60
60
Ctr
SP-A
Surface GluA1 synaptic intensity (normalized)
1.5
1.0
0.5
0.0
59
59
Ctr
SP-A
M
Synapse density (normalized)
1.5
1.0
0.5
0.0
60
60
Ctr
SP-A
Surface GluA1 - DIV 15 hippocampal neurons
N
Surface GluA1 intensity (normalized)
1.5
1.0
0.5
0.0
***
**
*
*
51
50
51
36
22
21
21
- + + + - + +
:KD
- - + - - - -
:hGHS-R1a
- - - + - - -
:SP-A
- - - - - - +
:AZ

Supplementary Figure S 3.2 (Related to Figure 3.2) Ghrelin receptor constitutive activity regulates synaptic levels of GluA2, the regulation of GluA1 levels shows a dependence on the age of the cultures and the phenotype of the exposure to ghrelin receptor inverse agonist is similar to that of the knockdown of the ghrelin receptor

(A,B) Hippocampal neurons [15 d *in vitro* (DIV)] were incubated with ghrelin receptor inverse agonists [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-Substance P [SP-A] 1 μM (A) or AZ12861903 [AZ] 50 nM (B) for 20 h, and immunostained for surface GluA1 under non-permeabilizing conditions. After permeabilization, neurons were stained for MAP2, PSD95 and VGluT1 and analyzed for the total fluorescence intensity of GluA1 cell-surface puncta normalized to synapse density. Results are expressed as the percentage of control cells, and are averaged from 2–4 independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ by Mann-Whitney test. (C,D) Hippocampal neurons [15 d *in vitro* (DIV)] were incubated with the ghrelin receptor antagonist JMV2959 100 μM for 20 hours and immunostained for surface GluA1 under non-permeabilizing conditions. After permeabilization, neurons were stained for MAP2 and VGluT1 and analyzed for the total fluorescence intensity of GluA1 synaptic cluster (VGluT1-colocalized) per synapse density. Results are expressed as the percentage of control cells, and are averaged from 2 independent experiments. (E-M) Hippocampal neurons 15 DIV (E), 20 DIV (H) and 7 DIV (K) were incubated with the ghrelin receptor inverse agonist SP-A 1 μM for 20 h and immunostained for surface GluA2 (E) or GluA1 (H,K) under non-permeabilizing conditions. After permeabilization, neurons were stained for MAP2, PSD95 and VGluT1 and analyzed for the total fluorescence intensity of GluA2 or GluA1 cell-surface puncta and total fluorescence intensity of GluA2 or GluA1 synaptic clusters (VGluT1/PSD95-colocalized) per synapse density (F,I,L). Neurons were also analyzed for synapse density (VGluT1 puncta positive for PSD95) (G,J,M). Scale bar represents 5 μm. Results are expressed as % of control cells, and are averaged from 4–5 independent experiments. Error bars represent SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ by Mann-Whitney test. Number in bars indicates number of cells analyzed for each condition. (N) Hippocampal neurons transfected at 7 DIV with Luciferase shRNA-GFP (control), GHS-R1a shRNA-GFP (KD), GHS-R1a shRNA-GFP + hGHS-R1a (rescue) or with GHS-R1a shRNA-GFP and treated at 15 DIV with SP-A or AZ were analyzed for the total fluorescence intensity of GluA1 cell-surface puncta normalized to synapse density. Results are expressed as % of control and are averaged from 3–4 independent experiments. Error bars represent SEM. Number in bars indicates number of cells analyzed for each condition. The statistical significance was calculated using the Kruskal-Wallis test followed by the Dunn's Multiple Comparison test ($^{**}P < 0.01$ and $^{***}P < 0.001$).

4.3 Neuron cultures previously exposed to ghrelin receptor inverse agonist show increased surface and synaptic GluA1 levels when exposed to ghrelin receptor agonist

Figure S3



Supplementary Figure S 3.3 After exposure to an inverse agonist of the ghrelin receptor, surface and synaptic GluA1 expression is increased by the administration of a ghrelin receptor agonist (A-C) Hippocampal neurons 15 DIV were incubated with the ghrelin receptor agonist MK-0677 (1 μM) for 1 h, with the inverse agonist SP-A (1 μM) for 15 min, or with the inverse agonist SP-A (1 μM) for 15 min followed by the ghrelin receptor agonist MK-0677 (1 μM) for 1 h. Neurons were live stained for GluA1 using an antibody against an extracellular epitope in the GluA1 N-terminus. After fixation, neurons were stained for PSD95, VGluT1 and MAP2. Scale bar represents 5 μm. Neurons were analyzed for the total fluorescence intensity of GluA1 cell surface (B) and GluA1 synaptic clusters (VGluT1/PSD95-colocalized) (C) per synapse density. Results are expressed as % of control cells, and are averaged from 3–5 independent experiments. Error bars represent SEM. The statistical significance was calculated using the Kruskal-Wallis test followed by the Dunn's Multiple Comparison test (*P < 0.05, **P < 0.01 and ***P < 0.001). Number in bars indicates number of cells analyzed for each condition.

5 Discussion

The constitutive activity of the ghrelin receptor has been previously described to regulate food intake and body weight (Fernandez et al., 2018; Petersen et al., 2009), but its involvement in the regulation of memory and learning has not been directly addressed. Here, we provide the first evidence that the ligand-independent activity of the ghrelin receptor regulates AMPA receptors at the synapse and the formation of spatial memories.

We found that the constitutive activity of the ghrelin receptor is important for both recognition memory, as assessed in the novel object recognition test, as well as spatial memory, evaluated in the object displacement test. We did not find alterations in anxiety levels upon administration of the inverse agonist of the ghrelin receptor. The effects of the ghrelin inverse agonist on memory that we observed are in agreement with the observation that ghrelin receptor knockout mice perform poorly on memory tests (Albarran-Zeckler et al., 2012; Davis et al., 2011), and suggest a clear role in memory for the usually high constitutive activity of the ghrelin receptor, an intrinsic feature of this receptor (Damian et al., 2012).

In order to understand the molecular mechanism through which the constitutive activity of the ghrelin receptor regulates memory, we tested whether inverse agonists of the ghrelin receptor affect the synaptic content of AMPA receptors, which are critical for synaptic plasticity processes thought to underlie learning and memory (Huganir and Nicoll, 2013). Object location memory is encoded, consolidated and retrieved by the hippocampus (Barker and Warburton, 2011; Broadbent et al., 2004; Mumby et al., 2002). Given that blockade of the ligand-independent activity of the ghrelin receptor severely impairs this type of spatial memory, we tested for effects in hippocampal neurons. We observed that the GHSR constitutive activity regulates the levels of synaptic GluA1 and GluA2 AMPA receptors in cultured hippocampal neurons, and found that the GHSR inverse agonist decreases the AMPA/NMDA ratio in hippocampal organotypic slice cultures. In agreement with these observations, we found a decrease on the synaptic levels of GluA1 upon silencing of the expression of the ghrelin receptor. We also confirmed the specificity of both inverse agonists of the ghrelin receptor, since they showed no effects on GluA1 in neurons depleted of the ghrelin receptor expression.

We further explored how the GHSR constitutive activity impacts the synaptic and surface levels of AMPA-receptors, which are controlled both by exocytic/endocytic receptor traffic and by the recruitment to the synapse of extrasynaptic receptors preexisting at the neuronal plasma membrane (Opazo and Choquet, 2011). Using single particle imaging, we evaluated how the ghrelin receptor ligand-independent activity affects the cell surface diffusion of AMPA receptors, since this factor is critically important for synaptic communication and synaptic plasticity (Penn et al., 2017). We observed that blocking ghrelin receptor constitutive activity enhanced the mobility of synaptic GluA1 and GluA2 AMPA-receptors, and decreased the residence of GluA1 and GluA2 at synapses.

Ligand-independent activity of the ghrelin receptor may be required for synaptic plasticity processes. Indeed, using a neuronal culture model of cLTP we observed that both inverse agonists of the ghrelin receptor block LTP, as measured by the synaptic accumulation of GluA1. These observations suggest that the high constitutive signaling of the ghrelin receptor is producing a signaling set-point that regulates the levels of synaptic AMPA receptors and synaptic plasticity in the hippocampus, thus contributing to the formation of memories.

Finally, we found that the ligand-independent activity of the ghrelin receptor controls the GluA1 synaptic levels through the phosphorylation of GluA1 at serine 845. Consistently, blockage of the ghrelin receptor constitutive activity fails to modulate synaptic levels of GluA1 when it is mutated to a phosphodead or phosphomimetic form at serine 845. Phosphorylation of GluA1 serine 845 regulates extrasynaptic membrane trafficking of GuA1, and primes AMPA receptors for synaptic insertion upon the induction of synaptic plasticity (Oh et al., 2006). Interestingly, phosphorylation of this site is also required for retention of spatial learning (Lee et al., 2003). Together, our data point to a role of the constitutive activity of the ghrelin receptor in regulating GluA1 phosphorylation and extrasynaptic insertion as well as in regulating membrane diffusion of AMPA receptors.

Besides signaling in response to ghrelin, and in the absence of the ligand, the ghrelin receptor has recently been shown to modulate dopamine signaling through heterodimerization with dopamine receptors DRD1 and DRD2 (Kern et al., 2012; Kern et al., 2015). In the hippocampus, GHSR1a and DRD1 form heteromers that are activated by DRD1 agonists to induce intracellular Ca^{2+} mobilization, activation of early synaptic plasticity markers (e.g. exocytosis of GluA1-containing AMPA receptors), and to modulate memory (Kern et al., 2015). Importantly, the dopamine-induced effect on Ca^{2+} signaling is independent of the constitutive activity of the ghrelin receptor in the GHSR1a:DRD1 complex (Kern et al., 2015), suggesting the role of the constitutive activity of the ghrelin receptor on memory that we describe runs parallel to the effects of dopamine on memory through the ghrelin receptor.

Our data reveal a unique and previously unrecognized physiological role for the constitutive activity of the ghrelin receptor in the regulation of memory through the regulation of synaptic AMPA receptors. The recently described liver-expressed antimicrobial peptide 2 (LEAP2), which is an endogenous antagonist of the ghrelin receptor which is decreased in fasted animals (Ge et al., 2018a), may exhibit inverse agonist activity (M'Kadmi et al., 2019). The overall constitutive activity of the receptor also depends on its expression levels, which in turn change according to the animal´s feeding status (Kim et al., 2003; Petersen et al., 2009). These observations therefore reveal a novel mechanism by which the internal state of animals may exert control over cognitive processes. Moreover, this role of the ghrelin receptor constitutive activity should be taken into account when considering its inverse agonists as anti-obesity agents.

Chapter 4 – NPY and Social Hierarchy

The presented work is under preparation for submission as a research article:

Lara O. Franco*, Mário Carvalho*, Jéssica Costa, Pedro A. Ferreira, Joana R. Guedes, Renato Sousa, Mohamed Edfawy, Ana L. Cardoso, João Peça, Social subordination following early life stress is linked to enhanced inhibitory synaptic communication and increased Npy1r expression in the prefrontal cortex

* These authors contributed equally to the work

I contributed to the design, execution and analysis of the electrophysiology experiments. I also participated in the writing of the article

1 Introduction

For most social animals a key form of physical and psychosocial stress comes from the position an individual occupies in its social hierarchy (Sapolsky, 2005). One extreme example of this relationship is stress-induced death of male cockroaches persistently forced into subordination (Ewing, 1967). Originally defined in chicken as the “pecking order” (Schjelderup-Ebbe, 1922), it has now been observed that most social animals engage in contests and displays of dominance and subordination with their conspecifics to define social ranks. Additionally, there are also remarkable adaptations to social challenges even in less complex organisms, such as coalition forming by crabs to repeal higher ranked intruders (Backwell and Jennions, 2004), or the ability of fish to infer social rank from observation alone (Grosenick et al., 2007). Remarkable physiological adaptations may then reflect dominance via the display of anatomical cues such as badges (Tibbetts and Dale, 2004) or bright colors (Burmeister et al., 2005). As such, both physiology and behavior are bidirectionally shaped by social rank and dominance.

In the mammalian brain, the medial prefrontal cortex (mPFC) is implicated in social hierarchy behaviors (Wang et al., 2011b; Zink et al., 2008b) and is also a main target for the neuroendocrine system (Diorio et al., 1993). Upon activation of the hypothalamic-pituitary-adrenal (HPA) axis, the mPFC is affected by glucocorticoid signaling (Arnsten, 2015) and can undergo a striking remodeling that impacts decision-making (Dias-Ferreira et al., 2009). In humans, the mPFC is activated during the perception of social rank and injuries to this region produce deficits in social and moral reasoning (Anderson et al., 1999; Zink et al., 2008b). In rodents, lesions to the mPFC lower the social rank and induce deficits in recognizing social hierarchies (Holson and Walker, 1986; Yosida and Okanoya, 2012). Recent studies have also shown that manipulation of synaptic strength in the dorsal mPFC provides direct and bidirectional control over social dominance (Wang et al., 2011b). Together, this data indicates the mPFC is reciprocally involved in biological aspects pertaining to both control of social dominance and integration of the effects of stress response. However, due to the intricate relationship between social rank and stress, there are few studies dissecting how stress regulates social dominance, particularly, how induction of stress prior to an individual being placed in a hierarchy with unfamiliar individuals will shape future behavioral strategies in adulthood.

Here we demonstrate that early life stress (ELS) and adverse rearing conditions imparted by maternal separation, lead to changes in dominance and social behavior in adulthood that correlate with a lower social rank when mice are placed in a larger social hierarchy with unfamiliar conspecifics. We performed gene expression analysis, morphology and electrophysiology studies and uncovered a striking remodeling in prefrontal synaptic network function. Importantly, from a large global gene expression alteration induced by ELS, we highlight *Npy1r* as an element with expression levels inversely correlated with the social rank of mice. We validated the role of this gene for the observed electrophysiology phenotype by reversing this phenotype in ELS mice using pharmacologic manipulations of the NPY1R.

2 Materials and Methods

2.1 Electrophysiology Solutions

Extracellular solutions

N-Methyl-D-Glucamine-based artificial cerebrospinal fluid (NMDG-aCSF)

Component	*Concentration (mM)*
N-Methyl-D-Glucamine (NMDG)	198.86
KCl	2.5
$NaHCO_3$	26
NaH_2PO_4	1.25
Glucose	12.5
Thiourea	2
L-ascorbic acid	1
Na-pyruvate	3
$MgSO_4$	10
$CaCl_2$	0.5

Adjust pH to 7.4 with HCl. Correct osmolality to 300-310 mOsm/Kg. Filter and store at 4ºC.

Recording aCSF (aCSF)

Component	*Concentration (mM)*
NaCl	120
KCl	2.5
$NaHCO_3$	24
NaH_2PO_4	1.25
Glucose	12.5
$MgSO_4$	2
$CaCl_2$	2

Adjust pH to 7.4 with HCl. Correct osmolality to 300-310 mOsm/Kg. Filter and store at 4ºC.

Internal solutions

Cesium methanosulfonate (CsMe-Int)

Component	*Concentration (mM)*
$CsMeSO_3$	107
CsCl	10
NaCl	3.7
TEA-Cl	5
HEPES	20
EGTA	0.2
ATP magnesium salt	2
GTP sodium salt	0.3

Adjust pH to 7.3 with CsOH. Correct osmolality to 295-300 mOsm/Kg. Filter and store at 4ºC.

Potassium gluconate-based internal solution (K-Int)

Component	*Concentration (mM)*
K-gluconate	145
HEPES	10
Na-phosphocreatine	10
EGTA	1
$MgCl_2$	2
ATP magnesium salt	2
GTP sodium salt	0.3

Adjust pH to 7.3 with CsOH. Correct osmolality to 295-300 mOsm/Kg. Filter and store at 4ºC.

Cesium chloride internal (CsCl-Int)

Component	*Concentration (mM)*
CsCl	120
HEPES	2
Lidocaine N-ethyl chloride	5
$MgCl_2$	2
ATP magnesium salt	2
GTP sodium salt	0.4

Adjust pH to 7.3 with CsOH. Correct osmolality to 295-300 mOsm/Kg. Filter and store at 4ºC.

2.2 Animals and behavior

For matings, Adult C57Bl6/J females and males (~12 weeks old) obtained from Charles River Laboratories (Saint-Germain-Nuelles, France) were used. After mating, the pregnant female was single-housed, and the male had no contact with the offspring. Animals were maintained in a temperature (22°C) and humidity (60%) controlled facility, on a 12-hour light-dark cycle (lights on at 07:00 am), with food and water ad libitum. Cage bedding was changed twice per week. Animal maintenance, treatments and experimental procedures were conducted according with Animals Use and Care Guidelines issued by FELASA (Federation for Laboratory Animal Science Associations) and the guidelines and regulations approved by Portuguese – ORBEA (Local agency responsible for Animal Welfare of the University of Coimbra/CNC) and DGAV (Portuguese Regulatory Agency) – and European directives on animal welfare.

2.3 Early life stress

Early life stress was induced using an adaptation of a well-described maternal separation and unpredictable stress (MSUS) protocol. This protocol aims at reproducing human childhood neglect and abuse (Weiss et al., 2011). Pregnant single housed females were used. The day of birth was assigned as day 0. During the 2 postnatal weeks, maternal separation was performed daily for 3 hours during the dark cycle. During this period mother and pups were put in separate clean cages with bedding. Dam was maintained under controlled temperature and available food and water. Cages were placed side by side, allowing visual and olfactory contact. Separation timing and maternal stress type were unpredictable. Maternal stress was either 20 min of restraint in a Plexiglas tube or 5-minute forced swim in cold water (18°C). Control litters were undisturbed, apart from cage changes and weighting. MSUS and control cages were changed 2 times a week and pups were weighted on postnatal (PND) day 2, 7, 14 and 21. On PND 7, all pups were marked with paint on the paws in order to allow identification throughout experiments. Weaning of both MSUS and control litters was performed at PND 21. Only males were used. Animals were reared in cages of 4 animals, separated according to being control or MSUS, and animals of different litters were housed together. Testing started when the animals were 8 weeks old.

2.4 Behavior

Tests were performed during the non-active light cycle, between 8 am – 19 pm and the used mice were acclimatized in the behavior room for 1h before testing. Animals were not weight matched for social behavior tests. The analysis and quantification of all tests were performed by trained experiments, blinded to phenotype, and behaviors were monitored by direct observation or using automated video tracking (Ethovision or Observer XT, Noldus Information Technology).

2.5 Social dominance tube test

In this work, social dominance hierarchy ranks were assessed using the tube test (Wang et al., 2014; Wang et al., 2011a). Aggressive behavior is absent in this test. The tube consists of a transparent plexiglass tube of 33 cm with an inner diameter of 3 cm which enables an adult mice to walk through without being able to invert directions. Each test session starts by placing two mice, each at one end of the tube, and releasing them. The test ends when a mice exists the tube with all four paws outside for more than 4 seconds. The first animal to do so is considered the subordinate. The trials last for as long as necessary for one animal to exit the tube. Light intensity was kept at 20 lux and camera recordings were performed in a side-plane view. Between tests the tube was cleaned with 70 % ethanol and left to dry. The tube tests were organized following the round-robin social dominance tube test (RRSDTT) schedule laid out by van den Ber et al. (Van Den Berg et al., 2015). This schedule followed certain rules: 1 – encounters were only performed between non-cagemates (optional); 2 – at the end of each round-robin trial, each animal performed the test with all other non-cagemate animals (or all other animals including cagemates); 3 – the time interval between two encounters of one animal remained more or less constant among all animals; 4 – the number of encounters two opponent animals faced until they performed the tube test with each other was kept more or less the same; 5 – the number of times an animal was placed on each edge of the tube was kept the same; 6 – the round-robin schedule was randomized between trials. These criteria were fulfilled by making the schedules automatically using a costum-made schedule generator. In the case of controls versus MSUS, each RRSDTT trial was composed by 2 cages of each phenotype with 4 animals each. During a trial, 96 tube tests were performed during 2 consecutive days. In the case of wild-type only, each trial had a variable number of encounters and was also performed during 2 consecutive days. The winner and duration of the test was scored manually using the analysis software Observer XT 12.0 (Noldus Information Technology, Netherlands).

These non-cagemates schedules were aimed at stimulating more closely the social interaction of animals among big communities where animals interact sparsely with a big number of individuals apart from the ones in close proximity.

2.6 Brain tissue collection and DNA/RNA extraction

DNA and RNA was collected in order identify the molecular and genetic of ELS and social dominance hierarchies. We used 2 cohort of MSUS and control animals with 13-19 weeks of age and 1 cohort of wild-type animals with 24 weeks of age. For the extraction, animals were deeply anesthetized with isoflurane (Abbott, Illinois, USA) and sacrificed by decapitation. The brain was extracted and regions of interest collected (mPFC, amygdala, hippocampus, hypothalamus and striatum). The tissue was flash frozen in liquid nitrogen and stored at -80°C until further processing. Genomic DNA and total RNA were

purified from mPFC using the ALLPrep DNA/RNA/miRNA Universal Kit (Qiagen). DNA and RNA samples were eluted in 50 µL and 40 µL of water respectively, and their concentration was measured using a Nanodrop 2000 spectrometer (Thermo Scientific, Waltham, MA, USA). DNA and RNA was stored at -20°C and -80°C respectively.

2.7 RNA sequencing and transcriptomic analysis

RNA sequencing and transcriptome analysis was performed in mPFC of 3 adult MSUS and 3 controls. Quantity and quality of total RNA samples was assessed using the Experion RNA StdSens Analysis Kit (Biorad). Ribozero RNA Libraries were prepared from RNA samples and used for RNA sequencing (100bp Single Read Sequencing, ~20 million reads/sample) with Illumina 2500 HiSeq. RNA libraries and sequencing were performed by Mount Sinai Genomics Core Facility (Icahn Medical Institute, NY, USA).

RNA sequencing data analysis were performed on the Illumina platform BaseSpace platform (Illumina, CA), based on the Tuxedo protocol established by Trapnell et al 2012 (Trapnell et al., 2012). This protocol includes two analysis tools – TopHat (Trapnell et al., 2009) (http://tophat.cbcb.umd.edu/) and Cufflinks (Trapnell et al., 2010) (http://cufflinks.cbcb.umd.edu/). Reads for each treatment group (control and MSUS) were aligned to *Mus musculus* genome with TopHat tool. To create a transcriptome assembly for each experimental conditions the alignments were provided to Cufflinks. The resulting assemblies were merged to allow the determination of transcript expression levels for the two groups. Expression levels and statistics were calculated. The change rate between phenotypes is presented as fold change (log_2 scale). False discovery rate corrected P values (q-values), gene and transcript description (including names and genome locations), and false discovery rate (FDR) was performed using BaseSpace (Illumina, San Diego, CA).

2.8 Quantitative Reverse Transcription Polymerase Chain Reaction

RNA sequencing was validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) of genes of interest with GO categories of showing receptor activity relevant for synaptic transmission and behavior. RNA from Ctr, MSUS, and wild-types was extracted according to the aforementioned method. RNA samples were converted to complementary DNA (cDNA) using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal). Contamination with genomic DNA was prevented with on-column DNase I treatment during RNA extraction and qRT-PCR primers designed to be complementary to cross adjacent exons. cDNA was stored at -20°C. The mPFC expression of the following genes was assessed through qRT-PCR: Drd1a, Drd5, Grm2, Hrh3 and Npy1r. Hprt was used as the housekeeping gene. qRT-PCR was performed in an iQ5 thermocycler (BioRad, Hercules, Ca, USA). Primers were designed with Beacon Designer 7 software. Specificity of primers was verified

through melting curve shape, peak temperature and the amplification of a single product with the expected size.

A master mix was prepared with (1) iQ SYBR® Green Supermix (BioRad) (5µL per well), (2) both primers (0.5µL per well), and (3) complementary DNA (4µL, diluted 1:5 in RNase-free water). The thermocycling reaction was initiated with (1) the activation of Taq DNA polymerase at 95°C for 3 min, followed by (2) 45 cycles of a 10 sec-denaturation step at 95°C, a 30 sec-annealing step at the optimal primer temperature of annealing (55°C) and a 30 sec-elongation step at 72°C. The fluorescence was measured after the extension step by the iQ5 Multicolor Real-Time PCR Detection System (BioRad). After the 45 cycles of amplification, a melting curve protocol was performed with 1 min-heating at 55°C, followed by 80 steps of 10-sec, with a 0.5°C increment at each step. All reactions were performed in duplicate.

Cycle threshold values were established automatically by the iQ5 Optical System Software (BioRad) on the exponential phase and baseline was set to the same fluorescence value for every run. The Pfaffl method was used to quantify the relative gene expression of each sample, considering the amplification efficiencies for each target gene. Amplification efficiency was determined using the formula E=10(-1/S)-1, where S is the slope of the standard curve. Data for each sample was normalized to the endogenous reference gene (Hprt).

2.9 Primer list for qRT-PCR

Chrna5 FW CAGTTAGTGGACGTGGATGA, Chrna5 RV CCACCATAGTCATCAGGATTCC
Drd1a FW TCTCAGTCACTTTTCGGGGA, Drd1a RV CAGATCGGGCATTTGGAGAG
Drd5 FW CTATTTCCAGACCCTTCCGC, Drd5 RV AGTTGGACCGGGATAAAGGA
Grm2 FW CTCCAGTGATTATCGGGTGC, Grm2 RV AGGATAATGTGCAGCTTGGG
Hprt FW CCCTGGTTAAGCAGTACAGC, Hprt RV ATCCAACAAAGTCTGGCCTG
Hrh3 FW GTCAGTCACTCGAGCTGTCT, Hrh3 RV AGGCCCATACAGCAGGAAG
Npy1r FW TGCTACTTCAAGATATACATTCGC, Npy1r RV GGAGAGCAGCATGATGTTGA.

2.10 Preparation of synaptoneurosome fractions

Synaptoneurosome is a subcellular fraction of neurons, enriched in content from the presynapse (synaptosomes) and postsynapse (neurosomes). Synaptoneurosome fractions preparation was as adapted from previously described protocols (Yin et al., 2002). Adult MSUS and control male mice (21-23 weeks old) and wild-type mice (19-23 weeks old) were sacrificed deep anesthesia with isoflurane (Abbott, Illinois, USA) and decapitation. mPFC was dissected and stored at -80°C. Synaptoneurosomes were obtained following the protocol: mPFC homogenates were prepared by adding 2 mL Buffer A (*) to samples and homogenized in a glass/glass tissue homogenizer (2 mL Kontes Dounce Tissue

Grinder) with a small clearance pestle (0.025-0.076 mm), performing 30 strokes; The samples were centrifuged at 800g and 4°C for 3 minutes and the supernatant was collected, a volume of 200 µL stored at 4 °C; This supernatant was passed through a filter of 8 µm pore size (Millipore) and the flow-through was collected; a subsequent centrifugation step was performed at 10.000 g and 4°C for 15 minutes and the pellet was collected; This pellet was resuspended in 200 µL of cold 1x PBS and quantified. The samples were validated by western blot verification of enrichment of synaptophysin and PSD95 in synaptoneurosomes when compared with brain lysates.

Synaptoneurosomes and brain lysates were disaggregated with an ultrasonic bath and centrifuged for 5 min at 5000 g. The supernatant was recovered and stored at 4°C. Protein concentration was quantified using Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific). Samples were stores at -80°C.

2.11 Gel electrophoresis and western blot

4 µg of protein from whole cell brain lysates and synaptoneurosomes were resolved by SDS-PAGE in 8% polyacrylamide gels. Proteins were transferred to PVDF membranes Amersham Hybond (GE Healthcare Life Sciences) and, after blocking at room temperature (RT) for 1 h in 1x TBS with 5% milk and 0.1% Tween-20 (TBS-T), the membranes were incubated with primary antibodies, followed by washes in TBS-T and incubated with horseradish peroxidase (HRP)-conjugated donkey secondary antibody (anti-mouse or anti-rabbit, Jackson Immunoresearch). Immunostaining was visualized using ECL western blot substrate (GE Healthcare Life Sciences) on a Storm 860 Gel and Blot Imaging System (GE Healthcare Life Sciences). Primary antibodies used in western blotting experiments were the following: anti-NPY1R (STJ94548, 1:2500, St John's Laboratory Ltd), anti-PSD-95 (6G6-1C9, 1:2000; Cell Signalling Technology) and anti-synaptophysin (1:10000; Abcam). Anti-β-actin (A5441, 1:5000; Sigma-Aldrich) antibody was used as loading control.

2.12 Electrophysiology

Coronal acute brain slices containing the mPFC (between bregma +1.98 and +1.70mm) (351) were prepared for electrophysiology from adult mice (16 – 19 weeks old), by an experimentalist blinded to the genotype. Extracellular solutions were prepared the day before each day of experiment. The osmolarity of all extracellular solutions was adjusted to 300-310 mOsm except for the internal solutions based on potassium-gluconate (K-int), cesium methanosulfunate (Cs-int) and cesium chloride (CsCl-int), which were prepared with an osmolarity of 295-298 mOsm. Osmolarity was corrected for with the main ion of each solution. The pH of all extracellular solutions was adjusted to pH 7.4 with HCl or NaOH, and internal solution to pH 7.3 with KOH or CsOH.

Before brain dissection, mice were deeply anesthetized with isoflurane (Abbott, Illinois, USA) and perfused with an oxygenated (95%:5% O2:CO2 mix) N-Methyl-D-Glucamine-enriched artificial cerebrospinal fluid (NMDG-aCSF). The brain was quickly removed and glued to a vibratome support filled with ice-cold, oxygenated NMDG-aCSF. 300µm coronal slices containing the cingulate, prelimbic and infralimbic cortex were obtained using a vibratome (Leica VT1200s, Leica Microsystems, USA) and immediately recovered at 32°C for 8 minutes in NMDG-aCSF. 2 slices with these characteristics were obtained per animal. The slices were then transferred to a holding chamber that contained oxygenated artificial cerebrospinal fluid (aCSF) and were kept 1 h before the recordings.

For recording, the slices were moved to the recording chamber and perfused with oxygenated aCSF (2 to 3 mL/min) at 25°C. Pyramidal neurons were identified under infrared-differential interference contrast (IR-DIC) visualization. Cells were patched with borosilicate glass recording electrodes with an access resitance of 3-5MΩ (Science Products) and filled with internal solution. For the recording of mEPSCs the CsMe-Int solution was used. For the recording of spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs/sIPSCs) a K-Int solution was used. For the recording of individual sIPSC and mIPSCs a CsCl-Int was used. For each animal, one slice was used for simultaneous sEPSC and sIPSC recordings and another for AMPA mEPSC recordings. The used slice and the type of recordings was randomized each day. For the recording of sEPSC/sIPSC no drugs were added to the recording aCSF. For the recording of AMPA mEPSC TTX (1 µM), bicuculline (25µM) and APV (50µM) to isolate AMPAR-mediated mEPSC. For the recording of individual sIPSC, CNQX (10 µM) and APV (25 µM) was used, and for mIPSCs, TTX (0.5 µM) was used additionally. For sEPSC/sIPSC recordings the holding voltage was -55mV, in order to simultaneously isolate both types of events (Wuarin and Dudek, 1993). The holding voltage for every other recording was performed under of -70 mV.

For mIPSC and sIPSC recording under antagonism of NPY Y1 receptor, slices were kept at least 30 min in oxygenated aCSF containing 1 µM BIBO 3304 trifluoroacetate (Tocris), a highly selective NPY Y_1 receptor agonist. The recordings were performed in aCSF containing the same concentration of the drug. The concentration was chosen according to the literature (Hamilton et al., 2013).

After analysis, the exclusion criteria for recordings was access resistance over 25 MΩ and access resistance variation of more than 20%. Recordings were low pass-filtered at 2 kHz and digitized at 20 kHz. For each recording 30 events were analyzed, with the exception of sIPSC (8 consecutive events analyzed). Data was acquired with a Multiclamp 700B amplifier and Digidata 1550A (Molecular Devices Corporation) and analyzed using Clampfit 10.7 software (AxonInstruments). Data was organized for statistical analysis using a costume made Matlab script.

2.13 Statistical analysis

All graphs show average values ± sem, unless otherwise specified. To test for the normality of the data distributions we used Shapiro-Wilk normality test and D'Agostino & Pearson normality testFor normal data we used two-tailed unpaired Student's t-test, one-way or two-way ANOVA analysis followed by

either Bonferroni or Tukey post hoc test. For non-parametric tests, we used two-tailed Mann-Whitney test or Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. Multiple t-test comparisons applied to genome-wide expression data were adjust using a False Discovery Rate. Correlation of data were performed using Spearman correlation. Statistical significance was set at ***$p<0.001$, **$p<0.01$, *$p<0.05$. All statistical analysis was performed using Graphpad Prism 7.

3 Results

To investigate the critical brain regions and molecules that control social dominance hierarchies, we used an ELS model. Following this form of early life stress we observed that ELS animal show submissive behavior (**Figure 4.1**) in accordance with previously described (Benner et al., 2014a). Our inference on social hierarchy was observed using a round-robin tournament of tube test trials (Van Den Berg et al., 2015), where each animal performed the test with every other non-cagemate animal. The test was repeated 8 times with 8 control and 8 ELS animals each. This effect was also observed across 3 independent replications. The consistency of the submissiveness shown by ELS animals over the 8 trials also shows that this behavior is not learned but the result of features intrinsic to the animals.

3.1 Animals that endured ELS show persistent submissive behavior in adulthood

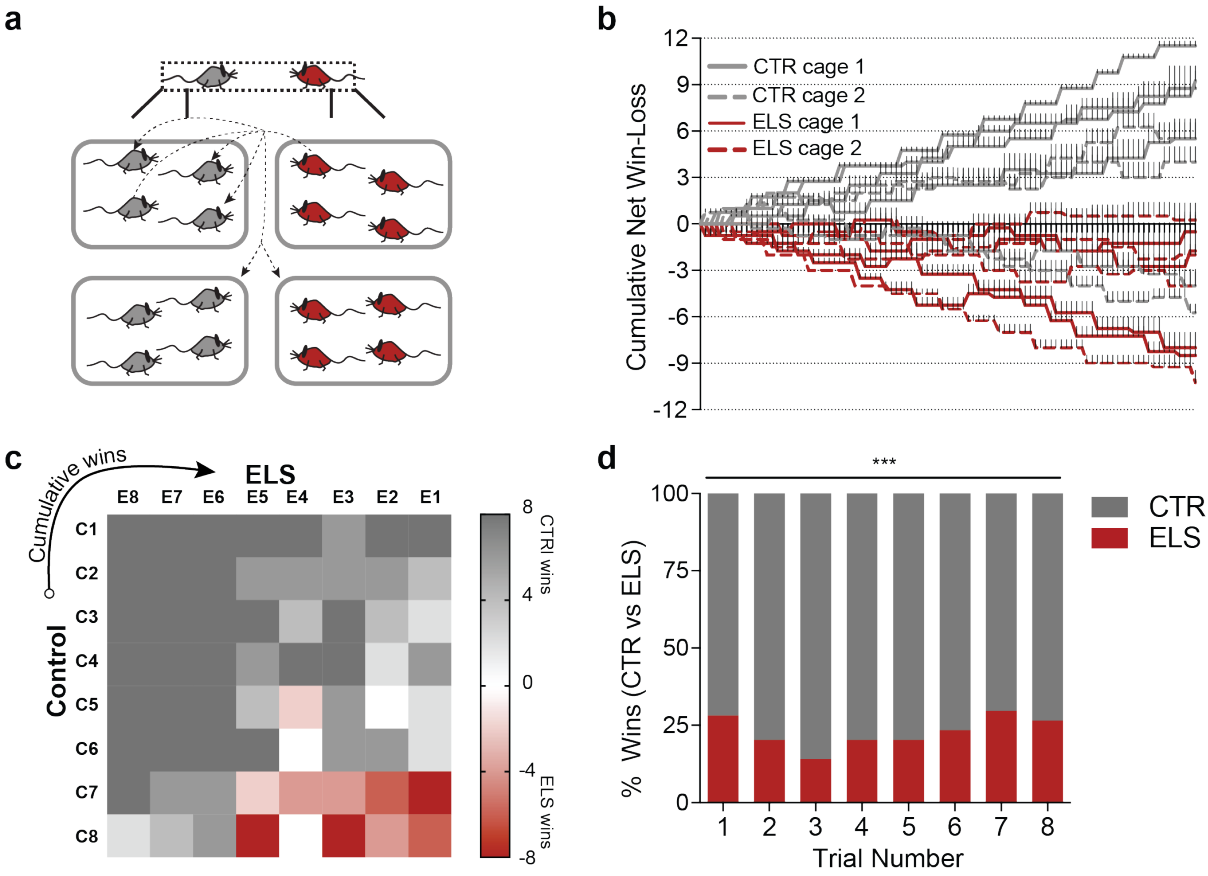


Figure 4.1 – Round-Robin Social Dominance Tube Test of control versus ELS animals shows that ELS animals are consistently submissive.
(A) Representation of the Round-Robin Social Dominance Tube test. (B) Hierarchy formed by non-cagemates ELS (n=8) and Ctr (n=8) after 8 trials. Each line represents the cumulative number of wins (+1) and losses (-1) and the corresponding rank obtained, for each animal. Each line shows mean and SEM. (C) Heatmap representation of all tube test results for ELS (n=8) and Ctr (n=8) after 8 trials. (D) Percentage of total wins for Ctr and ELS on each trial (ELS n=8, Ctr n=8, 8 trials). The results were

replicated in three independent cohorts. For statistical comparison two-way repeated measures ANOVA was used (*** $p<0.001$).

In our lab, we also observed this submissiveness of the ELS animals in the food competition test and in the social dominance tube test among cagemates (data not shown).

Using this model we asked whether brain regions known to be involved in social hierarchy where altered in their electrophysiology and molecular composition (**Figure 4.2**).We started by studying the electrophysiology of pyramidal neurons of the medial prefrontal cortex (mPFC), a region well known to be involved in social hierarchy (Wang et al., 2014; Wang et al., 2011a; Zhou et al., 2017). We performed this study in adult animals that had undergone round-robin social dominance tube tests. We observed similar frequency and amplitudes for spontaneous excitatory postsynaptic currents of ELS and Ctr mice (**Figure 4.2** A-E), however, we found that spontaneous inhibitory currents displayed higher amplitudes when comparing ELS to Ctr mice (**Figure 4.2** F-J).

3.2 Pyramidal neurons of the medial prefrontal cortex of ELS mice show increased amplitude of spontaneous inhibitory currents.

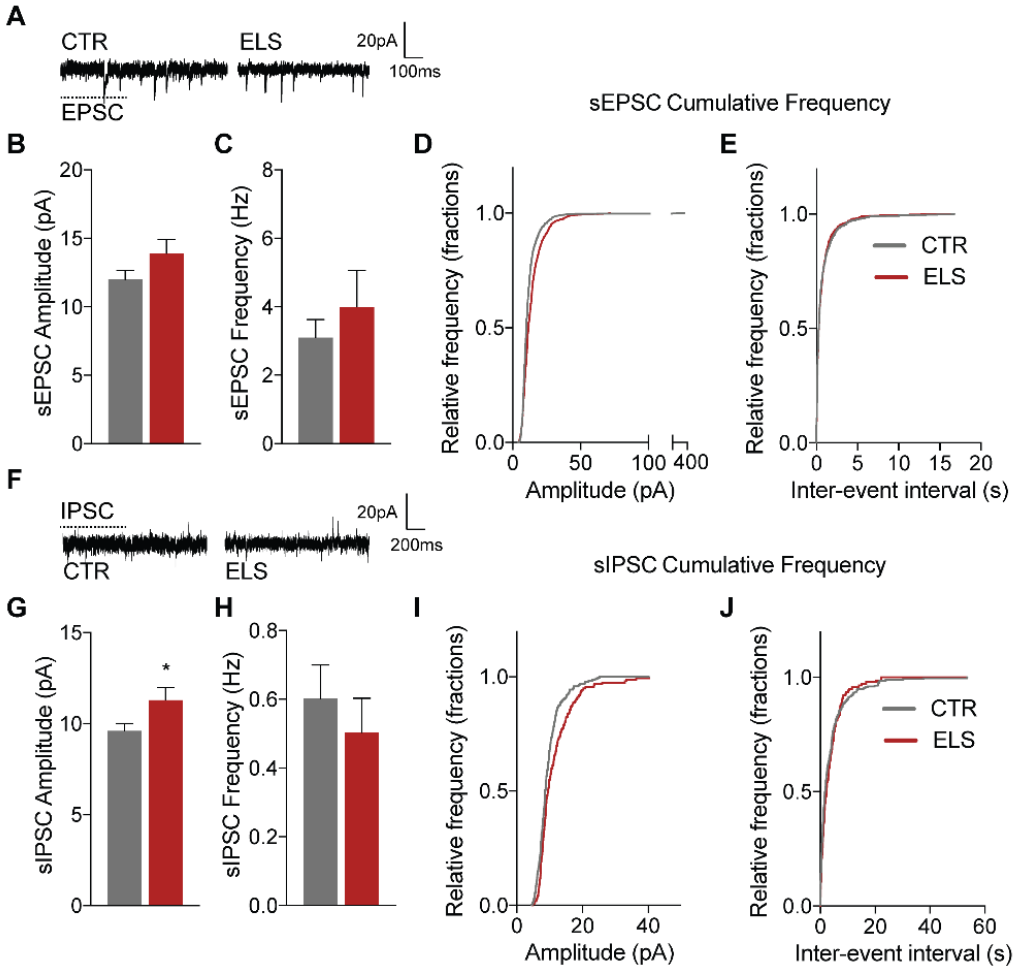


Figure 4.2 – Pyramidal neurons of the medial prefrontal cortex of ELS mice show increased amplitude of spontaneous inhibitory currents.
(A) Representative traces for spontaneous excitatory postsynaptic currents (sEPSC), scale bar 20 pA and 100 ms. (B) average amplitude and (C) average frequency of sEPSC, (D) cumulative distribution of the amplitude and (E) cumulative distribution of inter-event interval of every sEPSC event analyzed (control n=27 cells, ELS n=15 cells). (F) Representative traces for spontaneous excitatory postsynaptic currents (sIPSC), scale bar 20 pA and 200 ms. (G) average amplitude and (H) average frequency of sIPSC, (I) cumulative distribution of the amplitude and (J) cumulative distribution of inter-event interval of every sIPSC event analyzed (control n=35, ELS n=20). Statistical comparisons were performed using two-tailed unpaired t test (* $p<0.05$). Data are represented as means and SEM.

This effect may be attributed to an increase in the number of GABA receptors in postsynaptic terminals or an alteration of the kinetics of GABA receptors already present in the postsynaptic terminals. These observations are consistent with described alterations in the electrophysiology of the mPFC of animals that suffered ELS (Chocyk et al., 2013a; Chocyk et al., 2013b).

3.3 Antagonism of NPY1R ablates the observed increases in sIPSCs of pyramidal neurons in the mPFC of animals that endured ELS

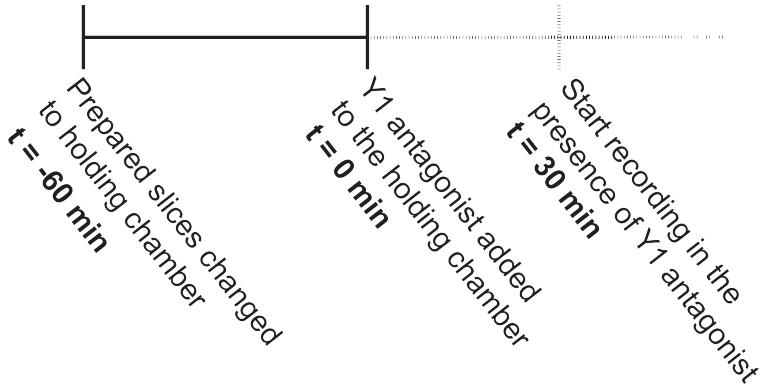


Figure 4.3 – Scheme of the experiment for the assessment of sIPSCs and mIPSCs in neurons of the prefrontal cortex of ELS and control animals. After slices were prepared using NMDG-based solutions, all slices were left to recover for 1h in recording aCSF (each day of experiment, slices were prepared in the morning from two animals in direct succession). At the end of this hour, the NPY receptor Y1 antagonist BIBO 3304 trifluoroacetate was added to the holding chamber containing all the prepared slices, with the final concentration of 1 μM. Slices were left at least 30 mins in the holding chamber with NPY receptor antagonist. After the period we started the recordings.

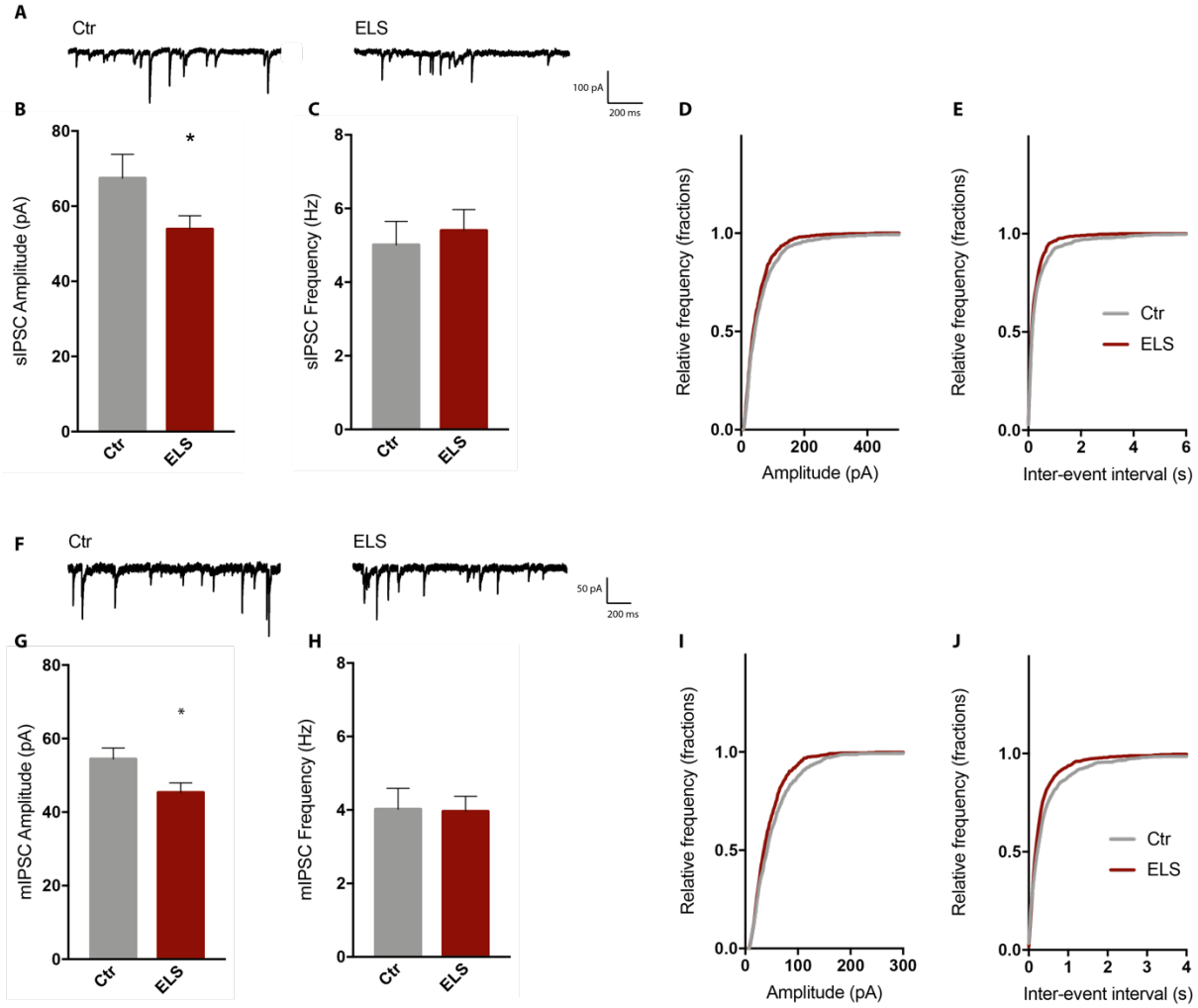


Figure 4.4 – Specific antagonism of NPY1R on mPFC neurons, using BIBO 3304 trifluoroacetate, ablates the observed effects of ELS on spontaneous inhibitory currents and significantly decreases the amplitude of mIPSC in ELS mice. (A) Representative traces for spontaneous inhibitory postsynaptic currents (sIPSC), scale bar 100 pA and 200 ms. (B) average amplitude and (C) average frequency of sIPSC, (D) cumulative distribution of the amplitude and (E) cumulative distribution of inter-event interval of every sIPSC event analyzed (control n=31 cells, ELS n=25 cells). (F) Representative traces for spontaneous excitatory postsynaptic currents (sIPSC), scale bar 50 pA and 200 ms. (G) average amplitude and (H) average frequency of sIPSC, (I) cumulative distribution of the amplitude and (J) cumulative distribution of inter-event interval of every sIPSC event analyzed (control n=28, ELS n=20). Statistical comparisons were performed using two-tailed unpaired t-test (* $p<0.05$). Data are represented as means and SEM.

We asked whether NPY1R was responsible for the observed functional differences in the electrophysiology of the mPFC of ELS mice (increased amplitude in spontaneous inhibitory current amplitude, **Figure 4.6** F-J). To this end, we prepared a new cohort of ELS and Ctr mice, administered 1µM of a specific NPY1R antagonist, BIBO 3304 trifluoroacetate, to brain slices containing the mPFC for at least 30 min and performed the recording under the same concentration (**Figure 4.3**). We observed an ablation of the observed effect on the peak amplitude of sIPSC, on pyramidal neurons of the mPFC, with no change in frequency (**Figure 4.4** A-E). We also observed a significant decrease in the amplitude of mIPSC with no change in frequency (**Figure 4.4** F-J). This observation confirms the

role of NPY1R on the previously observed effects. The induced decrease in mIPSC amplitude may be a result of an overreliance of the inhibitory gabaergic synapses on the increase in amplitude induced by the activation of this receptor (**Figure 4.2** F-J).

In order to confirm the molecular determinants of this effect on electrophysiology and behavior, we performed RNAseq of mPFC of both ELS and control mice (**Figure 4.5**). We detected a total of 13865 transcripts, of which 180 were significantly altered between experimental groups (**Figure 4.5** C). From these 180, 78 were up-regulated and 102 were down-regulated. A circus plot in **Figure 4.5** d shows the significantly altered genes, their fold change and relative abundance in Fragments per kilobase of transcript per Million mapped reads (FPKM – log10 base). The altered genes are known to be connected with myelination (the case of *Mag*, *Mog*, *Mal*, *Opalin* and *Plp1*), which is a process known to be perturbed in primate and rodent models of neonatal stress (Howell et al., 2013; Yang et al., 2016), and also proposed to be involved with chronic stress (the case of *Grm2*) (Cuccurazzu et al., 2013; Nasca et al., 2015). In order to select the genes than most likely explain the electrophysiological and behavioral findings, we performed a bioinformatic query for the genes that share three gene ontologies, “Biological Process – Behavior”, “Cellular component – Synapse” and “Molecular Function – Receptor Activity” (**Figure 4.5** E). From these we selected *Chma5*, *Drd1a*, *Drd5*, *Grm2*, *Hrh3* and *Npy1r* and validated the altered expression of these genes in new cohorts of Ctr and ELS mice using qRT-PCR (**Figure 4.5** E). We found a consistent significant increase in the expression of *Grm2* and *Npy1r* in ELS mice (**Figure 4.5** F).

3.4 Animals that endured ELS show increased expression of NPY1R mRNA in the medial prefrontal cortex

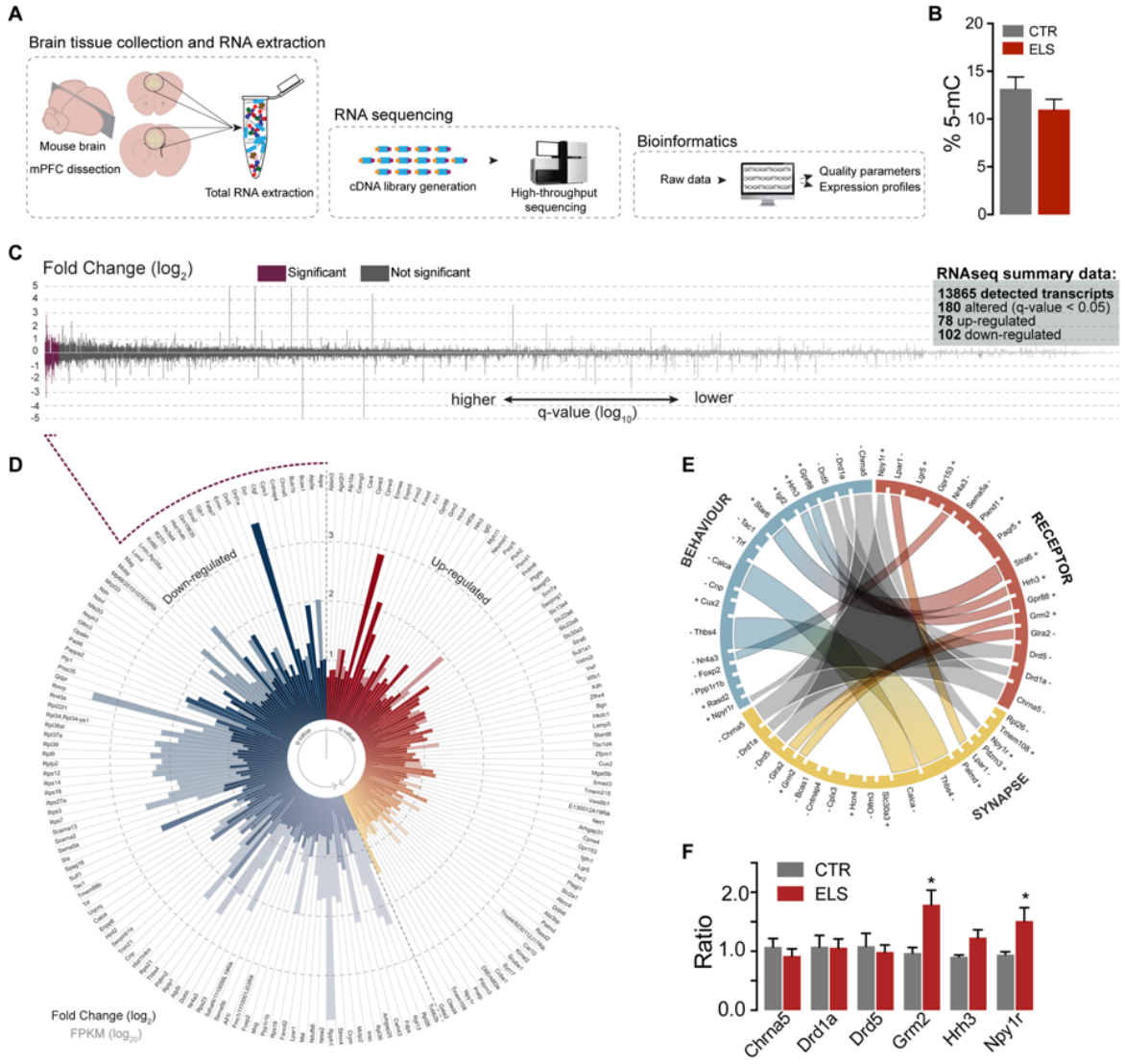


Figure 4.5 - The expression profile of the mPFC of ELS mice shows alterations in comparison to controls, and the NPY1R gene, among others, shows higher expression in ELS mice. (A) scheme of the collection and processing of samples for mPFC RNAseq. (B) Global DNA methylation levels are not significantly altered in the mPFC of adult male mice; CTR n=7, ELS n=9. (C) Representation of normalized fold change (log2 on y axis) and q-value (log10 on x axis) for all transcripts detected in the RNAseq of ELS and Ctr animals (total of 13865 transcripts). (D) Circos plot display of up-regulated (in red, total of 78) and down-regulated (in blue, total of 102) transcripts in ELS mice compared to Ctr mice. Solid bars represent fold change (log2) and transparent bars represent transcript abundance in Fragments Per Kilobase Million (FPKM in log10). (E) Chord diagram of genes belonging to the Gene Ontology categories, “Biological Process – Behavior”, “Cellular component – Synapse” and “Molecular Function – Receptor Activity”, grey chords connect genes belonging to the three categories. (F) Validation by qRT-PCR of genes involved in behavioral regulation, Ctr n=5-13, ELS n=6-18. * p<0.05 with FDR correction. Data is presented as means and SEM.

As mentioned in the introduction of this chapter, NPY1R is a NPY receptor with high expression in the cortex and NPY can also be found in this region, where it is expressed by interneurons and co-released with GABA (Hendry et al., 1984). This is a relevant gene for the observed alterations in electrophysiology and behavior for several reasons. It is involved in the modulation and amplification of inhibitory GABAergic inputs into pyramidal neurons (Bacci et al., 2002; Vollmer et al., 2016), it plays a role in the regulation of anxiety and feeding behavior (Heilig, 2004), and its orthologue found in *C. elegans* is involved in social behavior (de Bono and Bargmann, 1998a).

We then asked whether altered *Npy1r* expression in the ELS animals was a result of the stress protocol or it a more general molecular determinant of the social behavior connected to social dominance hierarchies. To this end, we conducted round-robin tournament social dominance tube test with non-ELS wild type male mice and segregated mice onto dominant and subordinate according to their cumulative number of wins and losses (**Figure 4.6**). We then assessed the expression of 6 of the candidate genes: *Chrna5*, *Drd1a*, *Drd5*, *Grm2*, *Hrh3* and *Npy1r*. We found that *Grm2* and *Npy1r* are significantly more expressed in the mPFC of subordinate mice (**Figure 4.6** C). Furthermore, The expression level of Npy1r was inversely correlated with the percentage of wins (**Figure 4.6** D) or, in other words, the more submissive the animal, the higher the expression of *Npy1r* in the mPFC.

3.5 Submissive wild-type mice show increased expression of NPY1R mRNA in the medial prefrontal cortex

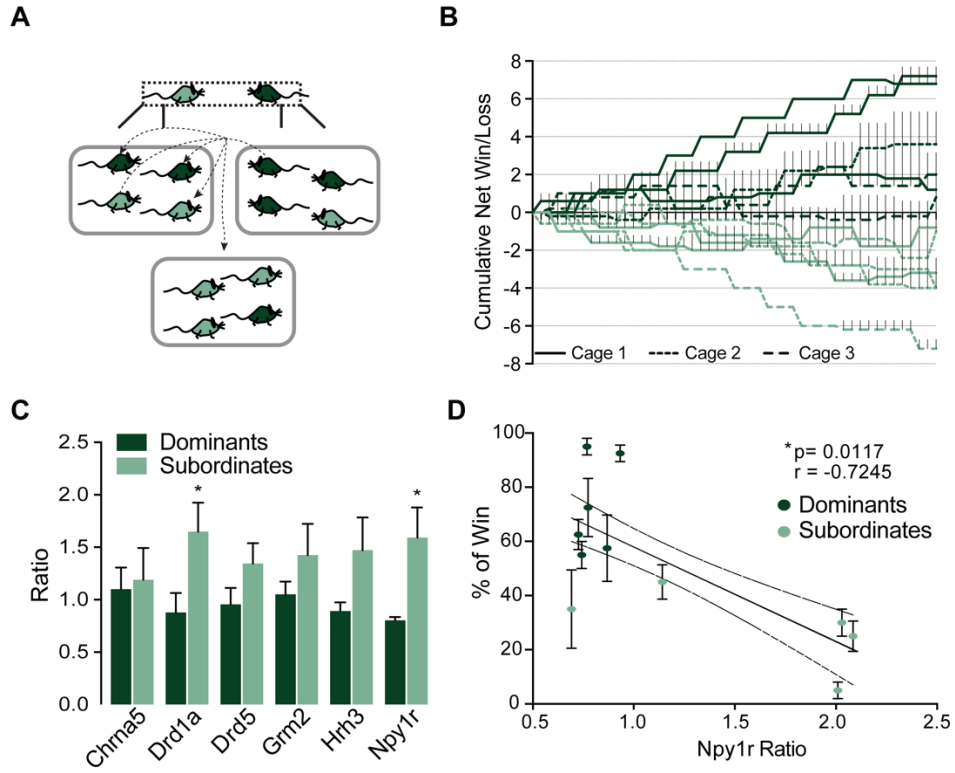


Figure 4.6 - Submissive mice show a significant increase in the expression of Npy1r *in mPFC.* *(A) Scheme of the* round-robin tournament social dominance tube test used in this experiment. Every mice performed the tube test with every other non-cagemate. (B) Hierarchy formed by non-cagemates Ctr (n=12) after 9 trials. Each line represents the cumulative number of wins (+1) and losses (-1) and the corresponding rank obtained, for each animal. Each line shows mean and SEM. (C) mPFC expression levels of 6 transcripts of interest in dominant (n=5) and subordinate (n=6) mice. Results are shown as qRT-PCR ratios for each transcript normalized for the internal control Hprt. (d) Correlation between Npy1r expression ratio and social dominance hierarchy rank after 9 RRSDTT trials. For statistical comparison we used two-tailed unpaired t-tests (C) and Pearson's correlation (D). * p<0.05, Data is presented as mean and SEM.

3.6 Submissive wild-type mice show increased expression of NPY1R protein in synapses of the medial prefrontal cortex

To assess if this increase in gene expression of *Npy1r* was also linked to an increased expression of the NPY1R receptor protein we generated a new cohort of wild type animals and segregated them as dominant and submissive according to their intra-cage social dominance hierarchy (**Figure 4.7** A). We then analyzed the expression of NPY1R in brain lysate and synaptoneurosome fractions (**Figure 4.7** B). We found no differences in the brain lysate fraction, and no differences in the postsynaptic marker PSD-95 (**Figure 4.7** C) and presynaptic marker synaptophysin (**Figure 4.7** D). These results lead us to conclude NPY1R expression is not only linked with early life stress but also with the level of submissiveness a mice manifests in its social dominance hierarchy.

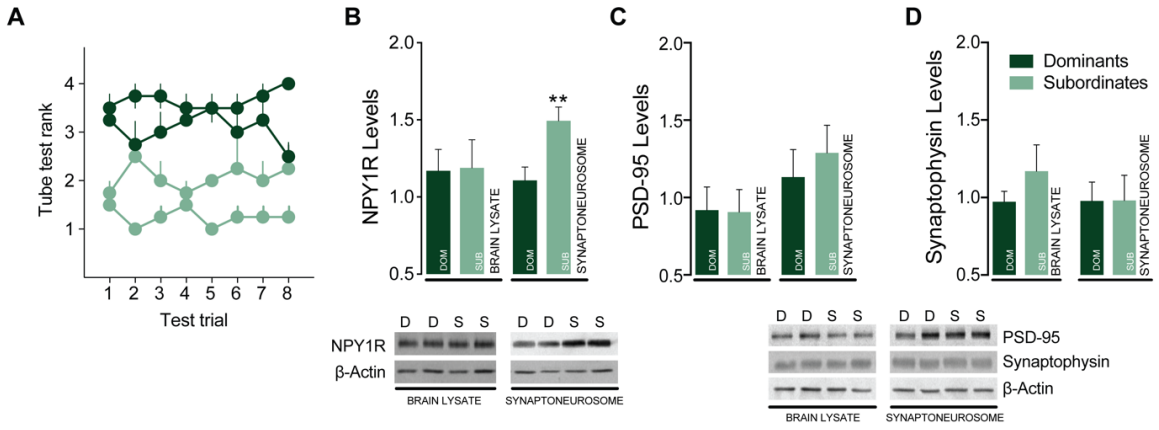


Figure 4.7 – NPY1R expression is increased in synaptoneurosome fractions of subordinate animals.
(A) Example of a social dominance hiearchy between adult wild type cage-mate mice (n=4, 4 mice per cage), points representing the average social dominance rank assessed for 8 days. Protein expression (assessed by western blot) of NPY1R (B), PSD-95 (C) and synaptophysin (D) in brain lysate and synaptoneurosome fractions of mPFC adult subordinate and dominant mice. D for dominant and s for subordinate. Statistical comparisons used two-tailed unpaired t-tests. * $p<0.05$, ** $p<0.01$. Data represented as mean and SEM.

3.7 Wild-type subordinate animals show decreased peak amplitude and increased frequency in sEPSC of medium spiny neurons of the nucleus accumbens.

We also asked what could be the consequences of the altered electrophysiology of the mPFC for downstream regions. Thus we performed sEPSC and mEPSC recordings in the nucleus accumbens of animals segregated as dominant and subordinate according to their intracage social dominance

hiearchies. These animals showed a decrease in peak amplitude and an increase in frequency of (sEPSC **Figure 4.8** A-E). No differences were observed in AMPA receptor-mediated mEPSCs (sEPSC **Figure 4.8** F-J). These results lead us to conclude that nACC medium spiny neurons of subordinate mice may have an increased number of active synapses with lower synaptic strength. Alternatively the circuit may be overall more excitable.

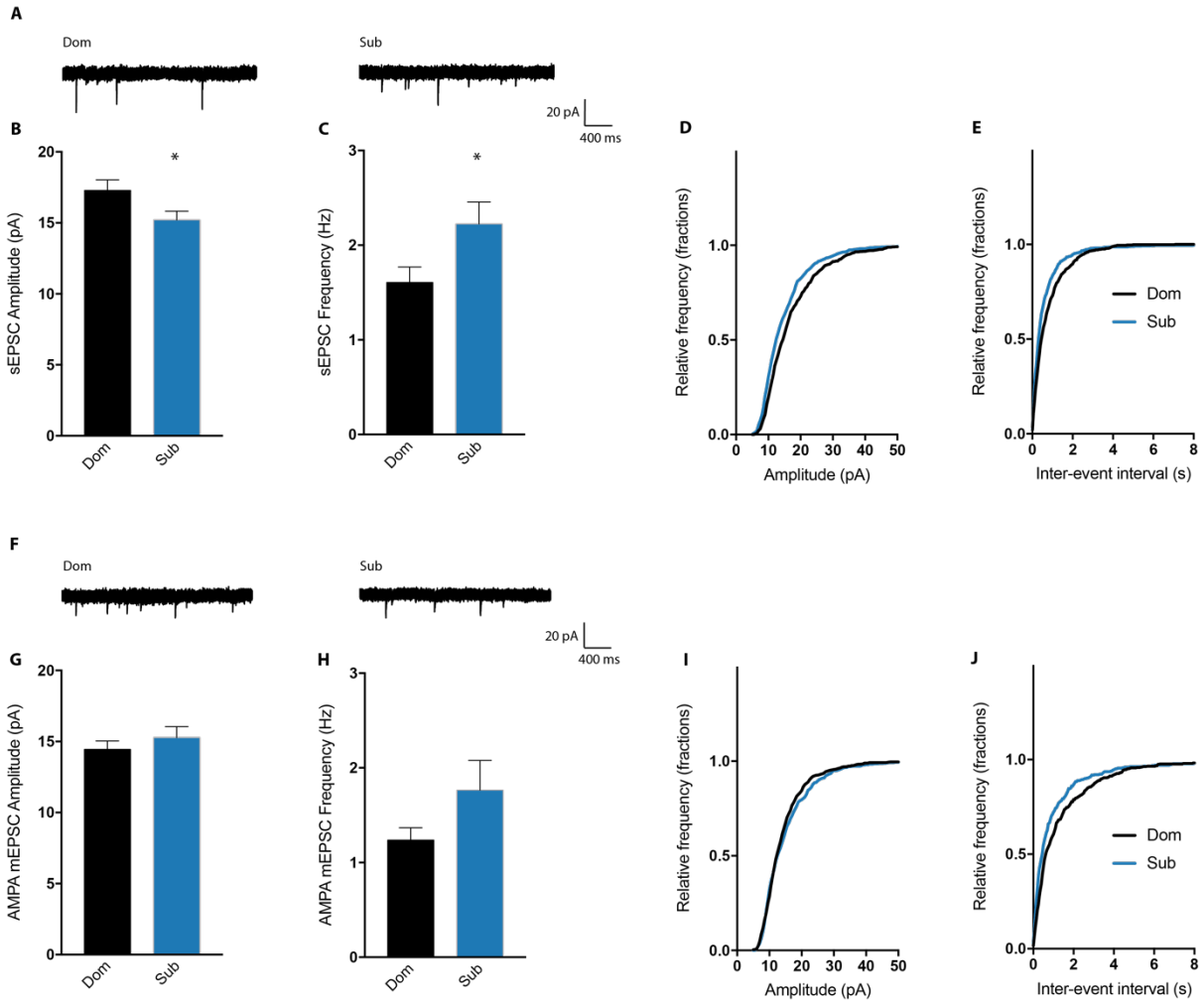


Figure 4.8 – Wild-type subordinate animals show decreased peak amplitude and increased frequency in sEPSC of medium spiny neurons of the nucleus accumbens.
(A) Representative traces for spontaneous excitatory postsynaptic currents (sEPSC), scale bar 20 pA and 400 ms. (B) average amplitude and (C) average frequency of sEPSC, (D) cumulative distribution of the amplitude and (E) cumulative distribution of inter-event interval of every sEPSC event analyzed (control n=23 cells, ELS n=28 cells). (F) Representative traces for AMPA receptor -mediated mini excitatory postsynaptic currents (AMPA mEPSC), scale bar 20 pA and 200 ms. (g) average amplitude and (h) average frequency of AMPA mEPSC, (i) cumulative distribution of the amplitude and (j) cumulative distribution of inter-event interval of every mEPSC event analyzed (control n=26, ELS n=22). Statistical comparisons were performed using non-parametric Mann-Whitney test (* $p<0.05$). Data are represented as means and SEM.

4 Discussion

We studied how the brain processes social dominance hierarchy information through the study of a model of early life stress. The choice of this model was based on the observation that it produces subordinate animals (**Figure 4.1**), as assessed not only with the tube test but also with food competition. Our work supports the previous findings of social subordination in mouse models of early life stress (Benner et al., 2014b; Benner et al., 2015), but diverges from work using a neonatal rat stress model (Tada et al., 2016a), which suggest that variations in species, the duration or intensity of stressors provokes different responses in individuals (Franklin et al., 2012; Krishnan et al., 2007; Sapolsky, 2015). Our ELS model was validated by the observation of increased depressive-like behavior, consistent with other studies (Franklin et al., 2010; Gapp et al., 2014; Lupien et al., 2009; Sandi and Haller, 2015) and altered performance in anxiety tests (results no shown). Using this model, we hoped to increase the likelihood of finding stronger molecular and brain region determinants of social dominance hierarchies and adult social behavior deficits associated with early life stress.

We questioned if we could identify the cellular and molecular markers of social subordination. At the genetic level, selective breeding programs have demonstrated that traits regulating social dominance may be rapidly enhanced in the span of only a few generations (Masur and Benedito, 1974; Moore et al., 2002), and genetic association studies have also suggested that traits influencing social dominance may be inherited (van der Kooij and Sandi, 2015). However, the identification of individual genes and directly determining their impact on cellular and synaptic network activity responsible for social dominance have not been widely studied (Robinson et al., 2005; van der Kooij and Sandi, 2015). Some examples include the serotonin transporter Slc6a4 knockout mice which display overtly submissive roles when pitted against wild type animals (Lewejohann et al., 2010). Also, in the dopamine transporter knockout mice (Dat1), social organizations are altered due to a reduction in behavior repertoires and a maladaptation to social contingencies (Rodriguiz et al., 2004). Dysfunctional dominance behaviors have also been linked to synaptic proteins SHANK3, Synapsin-II, Synapsin-III and Neuroligin-2 (Greco et al., 2013a; Nesher et al., 2015a; Zhou et al., 2016). To identify the molecular determinants of the alterations in electrophysiology, we performed RNAseq of this brain region and, in a more targeted manner, performed qRT-PCR for genes expected to be involved (**Figure 4.5**). We observed that the NPY1R and Grm2 genes were overexpressed in the mPFC of ELS mice, that the expression of NPY1R correlated with the level of submissiveness of hierarchized wild-type mice and that the corresponding NPY1R protein was over-expressed in the synapses of subordinate wild-type mice (**Figure 4.5** and **Figure 4.6**).

At the cellular level, we observed an increase in spontaneous inhibitory postsynaptic current amplitude, with no alteration in frequency, in pyramidal neurons of the mPFC (**Figure 4.2**). This can be interpreted as an increase in the sensitivity to GABA or postsynaptic GABAergic terminals, as an increase in the presynaptic release of GABA, or both. The effects in this region are consistent with the described effects of chronic stress, during early childhood, on the function and anatomy of the mPFC, hippocampus and amygdala (McEwen et al., 2016; Sandi and Haller, 2015). This indicates that NPY1R overexpression

may explain part of the electrophysiology and molecular alterations responsible for the submissiveness in social dominance hierarchy. We validated the role of NPY1R on the observed alterations in the electrophysiology of ELS mice by conducting similar experiments but now under specific antagonism of NPY1R (**Figure 4.4**). This manipulation ablates the observed effects on spontaneous inhibitory postsynaptic currents and further decreases mini-inhibitory currents amplitude without affecting their frequency.

Npy1r is an interesting gene because of its link with anxiety (Bertocchi et al., 2011b), foraging (Padilla et al., 2016) and regulation of social behaviors (de Bono and Bargmann, 1998b). We postulated that Npy1r could mediate behavioral adaptations linked to subordinate behavior and increased risk-taking behavior. Activation of NPY1R was previously shown to increase evoked inhibitory postsynaptic currents in prefrontal cortex pyramidal neurons (Bacci et al., 2002; Vollmer et al., 2016). Bacci and colleagues proposed that NPY1R was found in the presynaptic terminals of GABAergic synapses unto pyramidal neurons and that the activation of the receptor lead to an increase in calcium-dependent release of GABA (Bacci et al., 2002). Vollmer and colleagues also reported that the activation of NPY1R affects fear extinction recall (Vollmer et al., 2016). Activation of the NPY1R can be the result of NPY released from NPY-producing interneurons found inside the cortex or from projection of hypothalamic NPY neurons, although yet to be mapped (Hendry et al., 1984).

In other brain regions, it is reported that NPY1R activation in dendrites and soma leads to reduced cell excitability and reduced amplitude of evoked NMDA-mediated excitatory postsynaptic currents (Molosh et al., 2013; Sun et al., 2001). In our electrophysiology data we do not have clear indications that cell excitability is altered, since we did not observe shifts in the frequency of spontaneous activity. Nevertheless, this question may be answered in the future by performing current clamp recordings of resting state membrane voltage.

We also observed that the electrophysiology of the nucleus accumbens is altered in subordinate mice. In terms of information flow, this is a downstream region from the medial prefrontal cortex (Zhou et al., 2017). The nucleus accumbens is highly involved in social behavior and has already been implicated in submissiveness (Goette et al., 2015; Hollis et al., 2015; van der Kooij et al., 2018). A decrease in amplitude with increases in frequency of spontaneous excitatory postsynaptic currents suggests that subordinate animals receive a higher number of low strength inputs into medium spiny neurons. This observation can be interpreted as an increase in the number of immature synapses onto these neurons or a decrease in the number of silent synapses. It should be noted that the prefrontal cortex is not the only brain region that enervates the nucleus accumbens. Confirmation of the origin of these inputs can be done by imaging techniques and genetic marking of specific inputs. Zhou and colleagues explored the upstream regions from the mPFC involved in social dominance hierarchies and found that increasing the inputs from the mediodorsal thalamus is sufficient to increase the hierarchical position of animal (Zhou et al., 2017).

Using a combination of techniques, this work identifies an adaptive behavioral strategy in animals suffering adverse rearing conditions and implicates NPY1R in dominance behaviors, suggesting this receptor as a potential target to mitigate the negative consequences of chronic social stress. Social dominance processing comprises numerous brain faculties (Qu et al., 2017). The organism has to be able to recognize sensory cues related with social hierarchy, create representations of its own position in the hierarchy and the positions of other organisms with which it interacts, and generate and execute behavior plans accordingly (Qu et al., 2017). This work constitutes an effort to identify the minimal, most relevant brain regions and molecules that provide a top-down control over social dominance, in the expectation that these determinants can be exploited to better treat health problems connected with social dominance hierarchies. Social dominance hierarchies are also very interesting biological processes in that they can be clearly delineated and manipulated through behavior paradigms; the dynamics of these relationships can be represented by mathematical models, which can then be validated. This enables a potential avenue for the mathematical interpretation of behavior and the neuropeptide neuromodulation associated with it.

Chapter 5 – General discussion and further work

1 Signaling through ghrelin receptor constitutive activity

We showed that systemic administration of a blood brain barrier crossing inverse agonist of the ghrelin receptor prevents memory formation of the novelty and location of objects. This indicates that the activity generated by the receptor at a basal level is important to maintain these types of memory, on an acute timeframe. Memory for object novelty is known to be affected in the knockout model for ghrelin peptide (Diano et al., 2006b) and this type of memory is enhanced by systemic (Diano et al., 2006b) and ICV (Carlini et al., 2008) administration of ghrelin. Memory of object displacement has also been linked to prolonged administration of ghrelin (Kent et al., 2015). Since memory of object location is associated with hippocampal function (Bekinschtein et al., 2014) and the memory of object novelty is also the result, at least partially, of hippocampal function (Gerlai and Roder, 1995; Reed and Squire, 1997) we studied the molecular mechanisms behind this regulation of memory and the targeted effects of the inverse agonist in the hippocampus. This choice is also substantiated by the body of evidence linking the effects of ghrelin receptor activation, on memory enhancement, to the effects of the drug in the hippocampus (Carlini et al., 2010; Carlini et al., 2002a; Diano et al., 2006a; Ribeiro et al., 2014).

We performed targeted administration of the inverse agonist directly into the hippocampus. We chose to place the canula right above the dentate gyrus in order to inject the drug in a central position within the hippocampus and to affect primarily the dentate gyrus, a region suggested to have the highest expression of the receptor within the hippocampus (Diano et al., 2006b; Zigman et al., 2006). Although without statistical significance, we show that local blocking of the constitutive activity of the ghrelin receptor in the hippocampus has a tendency to reduce contextual fear memory. This result tends towards an agreement with the known effects of ghrelin on the enhancement of passive fear memory, and the link between the hippocampus and this effect (Carlini et al., 2004b; Diano et al., 2006b). With intrahippocampal administration we did not observe a clear effect on the novel object and object displacement tasks. This may be due to the increased reliance of these tests on low levels of anxiety of the animal or on the necessity of further adjustment of the drug dosage (Diano et al., 2006b). For instance, Diano and colleagues observed that the increased retention on T-maze footshock avoidance after ICV injections of ghrelin did not scale linearly with the concentration of ghrelin administered but had an inverted U-shape response profile. It is also possible that the observed effects of the systemic administration of the inverse agonist are only partially the result of its activity on the hippocampus. An alternative candidate region is the amygdala. Direct administration of ghrelin into the amygdala can also improve passive avoidance fear memory (Carlini et al., 2004b) and spatial learning (Toth et al., 2010). This question may be answered by probing the overall changes in brain activity after animals receive systemic inverse agonist doses and novel object and object recognition memory tests are performed.

We cannot exclude the fact that the inverse agonists used also possess considerable antagonistic activity at higher concentrations, and that this antagonist activity may contribute to the observed phenotype. Indeed, Beheshti and Shahrokhi showed that ICV administration of ghrelin receptor

antagonist impairs contextual fear memory (Beheshti and Shahrokhi, 2015) and Meyer and colleagues showed that systemic antagonism of the ghrelin receptor prevents stress-related enhancement of audiotory-conditioned fear memory (Meyer et al., 2014b). This is a common problem in the study of constitutive activity of GPCRs and can be solved by the development of more potent inverse agonists, in order to separate as far as possible the concentrations of maximal antagonism and inverse agonism action, and the precise charting of the pharmacokinetics and pharmacodynamics of these drugs. Ideally, this study could be conducted using transgenic models that express versions of the receptors that possess normal agonist activity and expression patterns but do not possess constitutive activity. In theory, this should be possible through the development of knock-in models that contain the human mutations known to ablate constitutive activity without affecting other functions of the receptor (Inoue et al., 2011; Pantel et al., 2006a).

Higher agonist-induced increase in the synaptic expression of the GluA1 after administration of inverse agonist suggests that blocking the constitutive activity of the ghrelin receptor also impairs its internalization. If we take into account the endogenous inverse agonists of the receptor, it is plausible that these not only serve to inhibit the constitutive activity of the receptor but also to enhance the activity of the endogenous agonists, by simply preventing the normal process of internalization of the receptor. The activity of other GPCRs that form heterodimerization complexes with GHSR1a may also be regulated in this manner.

The knowledge of the effects of constitutive activity of the receptor on memory and learning serve to warn of the implications of the usage of inverse agonists of the ghrelin receptor for the treatment of eating disorders, such as obesity. Furthermore, the endogenous regulation of this type of activity suggests new potential targets for the treatment pathologies connected with cognitive ability, such as Alzheimer's disease. The effects of the constitutive activity of ghrelin receptor on memory show that the information conveyed by neuropeptides can be transmitted by the mere existence of their receptors in the plasma membrane.

2 A connection between memory and feeding behavior

Feeding is one of the most universal and primitive abilities of organisms as it is tightly linked to survival. In the absence of pathology, most organisms experiencing hunger, when energy balance is low, proceed to forage for food. They can then store the foraged food and consume it until satiety. All these steps can be coordinated by the physiology of certain neuropeptides and hormones, and ghrelin has an important place among them. The memory enhancing function of ghrelin can provide a clear evolutionary advantage in that it enables organisms to better recall the location and condition of foraged food, especially when it is foraged under a state of hunger (Andrews, 2011). The constitutive activity of the receptor may act as a more long-term gain variable by maintaining and enhancing the memory and learning involved in this process. The recent discovery of endogenous mechanisms of direct regulation of this type of activity also open the possibility for more acute forms of regulation (M'Kadmi et al., 2019).

Dietrich and colleagues observed that the activation of the hypothalamic Agrp neurons not only increases feeding but, in the absence of food, decreases anxiety and increases stereotypical behavior (tested through marble burying), the latter in a manner dependent on the NPY5R activity (Dietrich et al., 2015). The authors suggested that this was evidence of a link between phylogenetically preserved functions (i.e. feeding) and more generalized functions, as those present in higher order organisms, suggesting that these general functions could also be coordinated by "lower" level neural circuits. This opens the possibility that the plethora of neuropeptides connected to feeding and the neural circuits in which they act could have their functions "abstracted" and re-utilized in other types of behavior. It is attractive to consider that the constitutive activity of the ghrelin receptor, through its function in memory, could also act in generalized forms of memory in higher organisms.

3 Activation of the NPY system as an adaptive strategy in dysfunctional rearing

We observed increased expression of NPY receptor NPY1R in the prefrontal cortex of animals that are subordinate following early life stress. NPY and its receptors are well known to reduce anxiety and increase resilience in stressed animals (Broqua, Wettstein et al. 1995)(Bertocchi et al., 2011a; Sajdyk et al., 1999). ICV injections of NPY into produce generalized anxiolytic effects (Broqua et al., 1995) and amygdalar injections increase social interaction (Sajdyk et al., 1999). The knockout for NPY1R receptor in the forebrain has increased anxiety, higher levels of plasmatic corticosterone and higher density of CRH fibers in the hypothalamus (Bertocchi et al., 2011a). In war veterans exposed to combat show high levels of plasmatic NPY if post-traumatic stress disorder was not developed (Yehuda et al., 2006). The highest levels were observed in those that undergone PSTD but recovered (Yehuda et al., 2006). These works suggest that NPY production is potentiated by prolonged exposure to stressors and improves the stress response of the organism, contributing to an adaptive interaction with the environment. This fact does not directly agree with our results. Assuming that the increased expression of the NPY1R in subordinate animals increases the action of NPY, we would expect these animals to be more dominant. We should take into account that the used social dominance hierarchy tests force all individuals to interact the same number of times. In a natural environment, subordinate individuals do not necessarily have dyadic encounters the same number of times as more dominant ones. The increased expression of NPY1R may be a mechanism of adaptation of already submissive individuals. We observed normal levels of plasmatic cortisol in ELS and control mice, decreased levels of anxiety and a decreased duration until resolution of dyadic encounters in animals that suffered early life stress (not shown). We propose that ELS favors adaptations that induce foraging and reduced competition within the social group. As summarized by E.O. Wilson from the works of Christian (Christian, 1970) and Calhoun (Calhoun, 1971), emigration and egress are tools of the juveniles and the subordinates, which makes these individuals the most likely to pioneer into new habitats in nature (Wilson, 2000). Nevertheless, it will be necessary to assess the behavioral effects of blocking NPY1R activity *in vivo* in subordinate animals.

There is a number of studies on the role of NPY and its receptors on the prefrontal cortex. Vollmer and colleagues observed that targeted injections of NPY into the infralimbic cortex in rats impaired the retrieval of extinction memory suggesting that higher NPY in this region prevents the formation of long lasting extinction of a previously conditioned averse stimulus (Vollmer et al., 2016). The administration of the Y_1 antagonist BIBO3304 was capable of reverting this effect. Humans with the polymorphism rs16147:T>C in the promoter region of the NPY gene have higher expression of the gene in the anterior cingulate cortex. These individuals also have increased propensity to anxiety and depression (Sommer et al., 2010). McGuire and colleagues also observed an increased expression and protein levels of NPY in the PFC of animals that underwent chronic variable stress (McGuire et al., 2011). They observed that this increase only happened after 7 days of the cessation of the stress protocol. Vollmer and colleagues also observed that the Y_1 receptor colocalized mainly with pyramidal neurons at the infralimbic cortex, which suggests, together with the described NPY-mediated inhibitory current potentiation, that these receptors are postsynaptic. This agrees with our observations.

We observed that animals that endured early life stress manifested increased amplitude of spontaneous inhibitory current in pyramidal neurons of the prefrontal cortex and that this effect could be reverted by the administration of a specific NPY1R receptor antagonist. These results suggest that the increased expression of NPY1R, and thus increased signaling by NPY, is responsible for the observed increase in inhibitory current amplitude. The dramatic effect of the administration of NPY receptor antagonist could be explained by a saturation of the signaling through the receptor. It is well described that administration of NPY to pyramidal neurons of the prefrontal cortex produces a sustained increase in the amplitude of evoked inhibitory post-synaptic currents (Bacci et al., 2002; Vollmer et al., 2016). The observed effects on the amplitude of events and not on their frequency suggest that NPY1R alters the post-synaptic activity of inhibitory synapses onto pyramidal neurons. This may happen through alteration of the number of GABAergic ion channels or the modification of their activity.

4 The role of neuropeptides in the maintenance of homeostasis

In this work we present new functions and mechanisms for a neuropeptide/peptide hormone canonically linked to feeding and a neuropeptide linked to feeding and emotional regulation. This work is the first to describe an effect of the constitutive activity of the ghrelin receptor in memory and learning and to describe a mechanism by which this effect may take place. This is also the first description of a connection between the NPY system and social subordination and the first suggestion that NPY could produce this effect through regulation of inhibitory currents in the prefrontal cortex.

Social behavior and feeding are also tightly connected, as argued in the general introduction. Very recently, Jennings and colleagues identified that the orbitofrontal cortex contains neuron ensembles that specifically fire in response to caloric and social stimuli (Jennings et al., 2019). The subsequent optogenetic control of these ensembles showed that the feeding neuron ensemble could drive an

increase in feeding and that the social stimuli-responsive neuron ensemble inhibited feeding. A similar approach could be used to find the link between social dominance hierarchy and feeding. Identification of the mPFC neural ensembles responsible for coding social hierarchy rank, a remarkable finding on its own, would enable the control of this behavior and to test whether they have a direct influence in feeding behavior. In his famous experiments, John B. Calhoun showed that in closed societies of rats a high enough population density leads to a dramatic decline in healthy social behavior and an eventual collapse of social order (Calhoun, 1971). Two striking changes in animal behavior that came with population density could explain the collapse. Rats began to eat only when in a group and would gather around only one of the many available food hoppers. Moreover, females decreased their ability to effectively rear their broods and stopped building nests. These observations suggest the importance of the interconnection between feeding, early life development and social behavior for the normal function of an organism in society. Understanding how these dimensions of behavior work together may have far reaching benefits in the treatment of eating disorders that have a strong social component, like obesity and anorexia, and may improve the understanding of the consequences of alterations of eating habits on the social behavior in the human society.

Chapter 6 - References

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