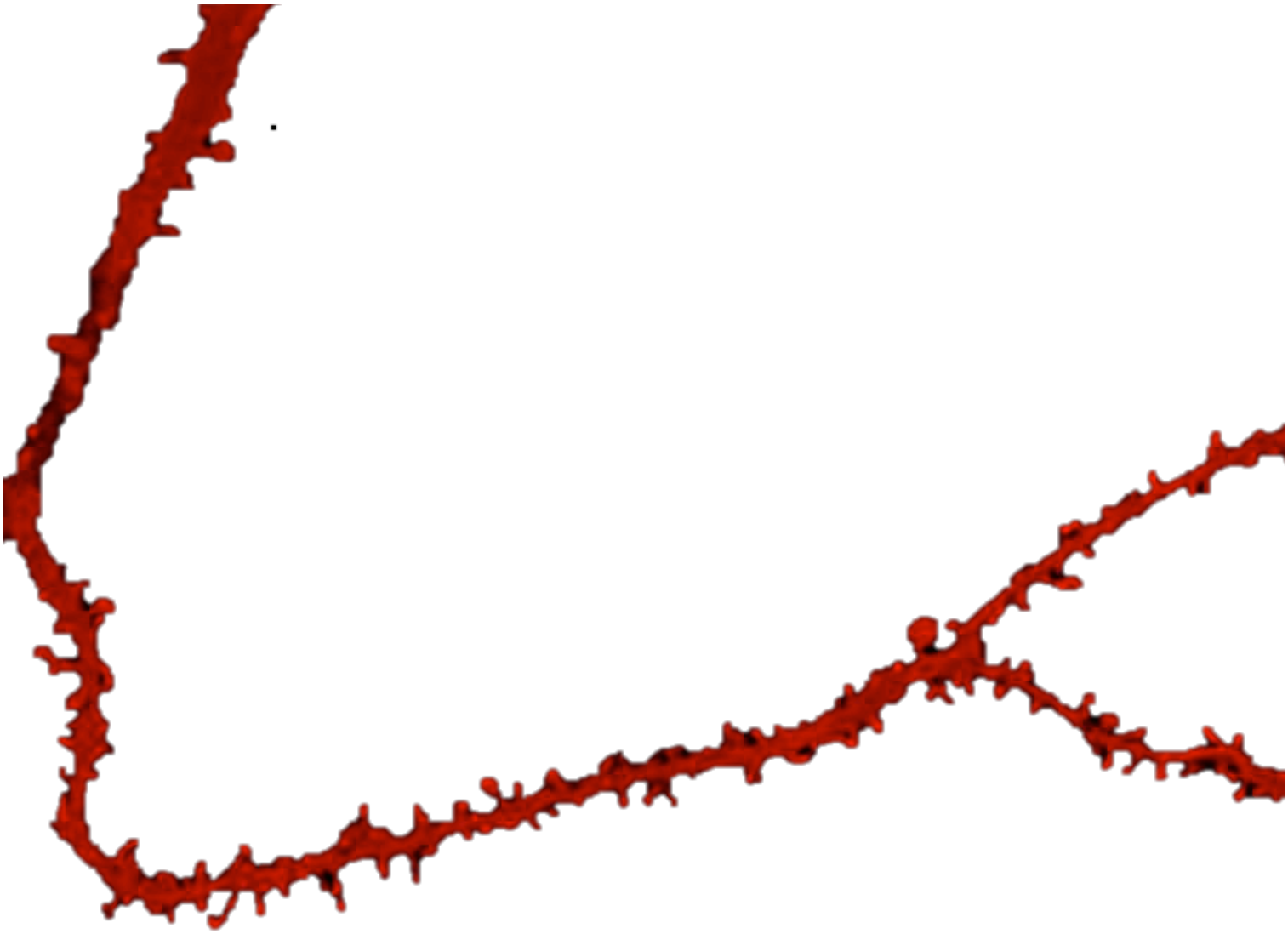

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Universidade de Coimbra

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

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Cover illustration: Dendritic spines from CA1 pyramidal neurons of hippocampal slices transfected with the *mcherry* construct

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ABBREVIATIONS

αMSH	alpha melanocortin
AC	adenylyl cyclase
ACC	acetyl-CoA carboxylase
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic-acid
AMPAR	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic-acid receptor
AMPK	5'-AMP-activated protein kinase
AgRP	agouti - related protein
ARC	hypothalamic arcuate nucleus
BBB	Blood brain barrier
CaMKK	calmodulin-dependent kinase kinase
cAMP	cyclic adenosine monophosphate
CREB	cAMP-response element binding protein
CRH	corticotropin-releasing hormone
DA	dopamine
DAG	diacylglycerol
DMH	dorsal medial hypothalamus
DRN	dorsal raphe nucleus
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
F-actin	filamentous actin
FAS	fatty acid synthase
GABA	γ-aminobutyric acid
GH	growth hormone
GHD	growth hormone deficiency
GHRH	growth-hormone- releasing hormone
GHS-R1a	growth hormone secretagogue receptor type 1a
GOAT	ghrelin O-acyl-transferase
GPCR	G protein–coupled receptor
GRIP	glutamate receptor-interacting protein
GSH	growth hormone segretagogue
IP_3	inositol-1,4,5-trisphosphate
Limk1	LIM domain kinase 1

LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
MK-0677	GHS-R1a agonist
NA	nucleus accumbeus
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NPY	neuropeptide Y
P-cofilin	Phosphorylated cofilin at Ser3
PI3K	phosphatidylinositol-3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
POMC	pro-opiomelanocortin
PSD	postsynaptic density
Q	glutamine
R	arginine
Ser	serine
SGZ	subgranular zone
SN	substantia nigra
SPAR	spine-associated Rap GTPase-activating protein
SV	synaptic vesicles
T-cofilin	total cofilin
VAMP	vesicle-associated membrane protein
VGCCs	voltage-gated calcium channels
VTA	ventral tegmental area

RESUMO

A capacidade de encontrar comida e de memorizar onde o alimento pode ser localizado novamente é crucial à sobrevivência. Portanto, a aprendizagem e a memória associadas à alimentação são processos críticos, em especial durante períodos de escassez de alimentos . A grelina é um peptídeo de 28 aminoácidos que estimula o apetite e que é produzida principalmente pelo estômago, sendo libertada na corrente sanguínea antes das refeições. A grelina exerce as suas funções através da ligação ao seu receptor, que é altamente expresso na hipófise, hipotálamo e hipocampo, e regula processos tais como o apetite, a ingestão de alimentos e a memória. O nosso laboratório mostrou que a grelina regula o tráfego sináptico de receptores de AMPA no hipocampo, produzindo modificações funcionais em sinapses excitatórias e aumentando a potenciação de longa duração (LTP), o correlato celular de aprendizagem e memória. Além disso, estudos anteriores mostraram que a grelina promove a formação das espículas dendriticas no hipocampo, e evidências preliminares indicam que a activação do receptor de grelina no hipocampo afecta a forma de espículas dendriticas. No entanto, o papel da grelina na regulação dinâmica das espículas dendríticas no hipocampo não é conhecido.

Para testar se a activação do receptor de grelina afecta as espículas dendríticas no hipocampo, desenvolvemos duas abordagens diferentes: Para mimetizar níveis de grelina elevados de forma prolongada, como ocorre durante o jejum, incubámos fatias de hipocampo organotípicas com o agonista do receptor de grelina, durante 20 h, e analisámos globalmente a morfologia das espículas. Por outro lado, para estudar os efeitos de uma elevação nos níveis de grelina num período curto, como ocorre durante os picos diários de grelina associados às refeições, estimulámos fatias de hipocampo organotípicas com o agonista do receptor de grelina, e acompanhámos individualmente as espículas durante um período total de 50 min. Enquanto a incubação prolongada com o agonista do receptor de grelina não teve impacto na morfologia geral das espículas, a análise das espículas individuais incubadas com o agonista por um curto período de tempo revelou um aumento progressivo do volume da cabeça das espículas em fatias, que atingiu significância estatística 50 minutos após a sua aplicação. Curiosamente, descobrimos que o efeito da activação do receptor de grelina sobre o volume das espículas depende do tamanho inicial da espícula. Nas espículas pequenas, a activação do receptor de grelina leva a um aumento de volume maior do que nas espículas maiores, para as quais a activação

do receptor de grelina impede as espículas de contrair. Para ambas as populações de espículas, a activação do receptor de grelina aumenta a fracção de espículas que crescem e diminui a população de espículas que contraem ao longo do tempo. Mecanisticamente, verificou-se que a activação do receptor de grelina, quer em fatias de hipocampo organotípicas quer em neurónios do hipocampo em cultura, conduz a uma acumulação de cofilina fosforilada, um regulador do citoesqueleto de actina das espículas.

Como um todo, este trabalho revelou pela primeira vez que a activação do receptor de grelina tem impacto na dinâmica das espículas de uma forma que é dependente do tamanho da espícula, e que se correlaciona com a fosforilação cofilina. Este efeito da ativação do receptor de grelina na dinâmica de espículas do hipocampo pode ter impacto na plasticidade estrutural das espículas em resposta à atividade, e pode estar na base dos efeitos da grelina no melhoramento cognitivo.

Palavras-chave: Grelina, Receptor da grelina, Hippocampo, Plasticidade estrutural, citoesqueleto de actina

ABSTRACT

The ability to find food and to remember where food can be located again is crucial for survival. Therefore, learning and memory associated to feeding are critical processes, in particular during periods of food shortage. Ghrelin is a 28-aminoacid appetite-stimulating peptide mainly produced by the stomach and released in the blood stream before meals. Ghrelin exerts its functions through binding to its receptor, the growth hormone segregatogue receptor type 1a (GSH-R1a), which is highly expressed in the pituitary, the hypothalamus and the hippocampus. Through activation of its receptor, ghrelin regulates processes such as appetite, food intake and memory. Our laboratory has shown that ghrelin regulates the synaptic trafficking of AMPA-receptors in the hippocampus, producing functional modifications at excitatory synapses and enhancing long-term potentiation (LTP), the cellular correlate of learning and memory. Moreover, previous studies showed that ghrelin promotes the formation of hippocampal dendritic spines, and preliminary evidence indicates that activation of the hippocampal GHS-R1a affects the shape of dendritic spines. However, the role of the ghrelin in regulating hippocampal dendritic spine dynamics remains unclear.

To test whether the activation of the GHS-R1a affects hippocampal dendritic spines we developed two different approaches: To mimic prolonged upregulated ghrelin levels, as occurs during fasting, organotypic hippocampal slices were incubated with the GHS-R1a agonist for 20 h, and overall spine morphology was analysed. On the other hand, to study short-term effects of ghrelin, expected to occur during the daily peaks in ghrelin associated with meals, we stimulated organotypic hippocampal slices with the GHS-R1a agonist, and followed individual spines for a total period of 50 min. Whereas long-term incubation with the GHS-R1a agonist did not have an impact on overall spine morphology, short-term incubation and time-lapse analysis of single spines revealed a progressive increase in spine head volume in slices incubated with the GHS-R1a agonist, which reached statistical significance 50 min after its application. Interestingly, we find that the effect of GHS-R1a activation on spine volume depends on the initial size of spines. In small spines activation of the GHS-R1a leads to larger volume increase than in larger spines, for which GHS-R1a activation prevents spine shrinkage. For both populations of spines GHS-R1a activation increases the fraction of spines that grow and decreases the population of spines that shrink throughout time. Mechanistically, we found that GHS-R1a activation either in organotypic hippocampal slices or in cultured hippocampal neurons leads to

an accumulation of phosphorylated cofilin, a major regulator of the spine actin cytoskeleton.

Altogether, this work revealed for the first time that GHS-R1a activation impacts spine dynamics in a manner that is dependent on spine size, and that correlates with cofilin phosphorylation. This effect of GHS-R1a activation on hippocampal spine dynamics may impact spine structural plasticity in response to activity, and may underlie the memory-enhancing effects of ghrelin.

Keywords: Ghrelin, GHS-R1a, Hippocampus, Structural plasticity, Actin cytoskeleton

Chapter 1

INTRODUCTION

HISTORICAL BACKGROUND

Endogenous oscillators mediate the pulsative release of hormones, including the release of growth hormone (GH) (Smith, Van der Ploeg et al. 1997). GH deficiency (GHD) is a medical condition, which comprises several symptoms including loss of bone mass, loss of musculature, poor memory and depression, and has also been pointed out as a potential cause for idiopathic short stature (reviewed in (Capatina and Wass 2015)). Due to the impact of the GHD on health, finding a synthetic molecule able to mimic or amplify the biological oscillator(s) that regulates GH-secretion, therefore being able to correct GHDs, has always been of big interest among the scientific community (Smith, Van der Ploeg et al. 1997).

More than two decades ago, it was developed the first synthetic peptides that were able to elicit the release of the GH, also termed as growth hormone segretagogues (GSH) (Momany, Bowers et al. 1981, Bowers, Momany et al. 1984, Smith, Van der Ploeg et al. 1997). These included GHRP-6, hexarelin, or the nonpeptide GHS MK-0677 (reviewed in (Kojima and Kangawa 2005)). However, at that time, a receptor that controlled GH release by interaction with those peptides was unknown. Only in 1996, Howard et al. identified a receptor for GHS termed growth hormone secretagogue receptor type 1a (GHS-R1a) (Howard, Feighner et al. 1996). This finding suggested that an endogenous ligand for GHS-R1a should exist and would regulate GH release in a different way from its regulation by hypothalamic GH-releasing hormones, such as somastatin, a peptide hormone that inhibits GH release (Brazeau, Vale et al. 1973). Three years later, Kojima and colleagues added different tissue extracts to cells expressing human GHS-R1a and found a major increase in intracellular calcium concentrations in the cells incubated with stomach extracts (Kojima, Hosoda et al. 1999). Through successive chromatography followed by amino-acid sequencing, the authors were able to isolate ghrelin, the native agonist for GHS-R1a (Kojima, Hosoda et al. 1999). Surprisingly, ghrelin was produced in the stomach before meals, being then released in the bloodstream (Cummings, Purnell et al. 2001). Studies using radiolabeled ghrelin also showed that ghrelin crosses the blood brain barrier (BBB) being able to reach the brain (Banks, Tschop et al. 2002). The discovery of ghrelin, its receptor and the studies that elucidated about its loci and its patterns of expression indicated that ghrelin was not only a GHS but could also exert other functions. Indeed, ghrelin was found to be extremely relevant in the regulation of food intake (Cummings,

Purnell et al. 2001, LeSauter, Hoque et al. 2009), a function that is consistent with its pattern of release before meals and has been involved in food intake, such that it is also known as “the hunger hormone”. Nevertheless, ghrelin also participates in the control of metabolism, arousal and even memory retention, mood and behaviour (Carlini, Monzon et al. 2002, van der Lely, Tschop et al. 2004). Peripheral metabolic hormones, such as leptin, affect several brain structures that are involved with endocrine and higher brain functions. As ghrelin integrates this category, there is a big interest in understanding ghrelin’s precise physiological role and mechanisms of function, other than a GHS.

GHRELIN

Ghrelin is a peptide hormone of 28-amino acids (Kojima, Hosoda et al. 1999) which concentration in the blood fluctuates throughout the day, rising before a meal and decreasing upon food consumption (Cummings, Purnell et al. 2001), as mentioned before. Ghrelin is mainly secreted by X/A-like cells in rats and P/D1 cells in humans of the oxyntic stomach mucosa (Date, Kojima et al. 2000, Ariyasu, Takaya et al. 2001), but was also found in other peripheral tissues such as the testis, ovary, placenta, kidney, pituitary, small intestine, pancreas, lymphocytes (reviewed in (Ferrini, Salio et al. 2009)). Circulating ghrelin is able to reach the brain because it crosses the BBB (Banks, Tschop et al. 2002), being able to bind to neurons of the hypothalamus, a region that is involved in the appetite control (reviewed in Ferrini, Salio et al. 2009). Thus, it is not surprising that ghrelin oscillations in the blood regulate food intake (LeSauter, Hoque et al. 2009). However, ghrelin is also highly expressed in the neurons, mainly in the neurons of the Arcuate Nucleus in the Hypothalamus (ARC), but the role of ghrelin produced in neurons remains less clear.

Ghrelin exists in two major forms: the acylated (acyl- or octanoyl- ghrelin) and the nonacylated (des-acyl ghrelin) form (Kojima, Hosoda et al. 1999, Hosoda, Kojima et al. 2000). The acyl ghrelin is originated by the attachment of a fatty acid side-chain (octanoyl group) at its third serine residue (Ser3). This posttranslationally modification is essential for binding to the GSH-R1a (Kojima, Hosoda et al. 1999) and is accomplished by the ghrelin *O*-acyl-transferase (GOAT), a member of the membrane-bound O-acyltransferase (Figure 1).

In humans, ghrelin gene is localized on chromosome 3, at position p25–26 (reviewed in (Kojima and Kangawa 2005)). The ghrelin gene originates two different transcripts, A and B, through alternative splicing mechanisms (Kojima and Kangawa 2005). Between the two, only

the transcript A encodes the ghrelin precursor protein, preproghrelin. In turn, preproghrelin will be further proteolytic cleaved to produce the acyl ghrelin form and other active peptide which function is poorly understood, the 23 amino acid peptide obestatin (Zhang, Ren et al. 2005, Seim, Amorim et al. 2010). The 28 amino-acid ghrelin is found in both human and rat and is the main form of the peptide (Kojima, Hosoda et al. 1999). In fact, the rat and human ghrelin differ in only two amino acid residues (Kojima, Hosoda et al. 1999). However, in the stomach of rodents, a second type of ghrelin peptide has been identified (Hosoda, Kojima et al. 2000). This peptide is similar to ghrelin, except for the deletion of the residue Gln14, therefore it was described as des-Gln14-ghrelin (Hosoda, Kojima et al. 2000). This type of ghrelin is also acylated, and has the same activities and potency as ghrelin but its levels in the rodents stomach is low (Hosoda, Kojima et al. 2000). As des-Gln14-ghrelin is not present in humans and as its levels of expression in rodents are low, the majority of studies focus on the study of the 28-aminoacid ghrelin.

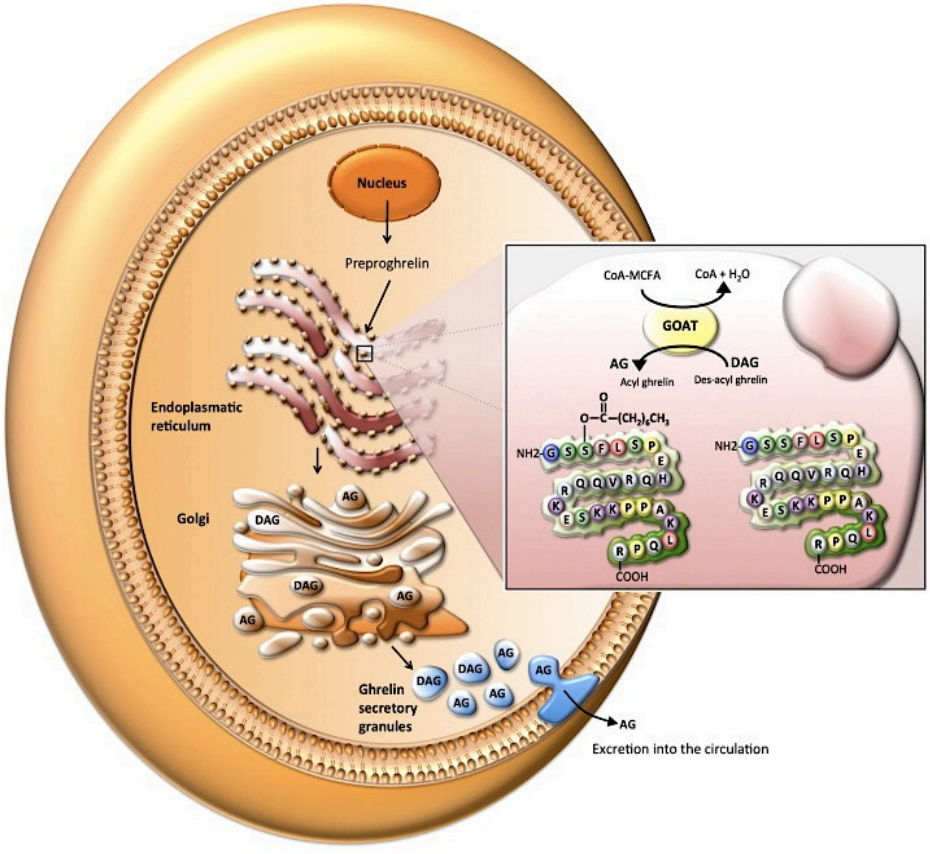


Figure 1. Schematic on the post-translational processing and acylation of ghrelin. Ghrelin is encoded by the preproghrelin gene, which also encodes for a small peptide termed obestatin. Ghrelin needs the enzyme GOAT to be acylated, a step essential for binding to the GHS-R1a receptor and exert its functions. Adapted from (Muller, Nogueiras et al. 2015).

GHRELIN RECEPTOR

Ghrelin acts via its receptor named GHS-R1a, which is a G protein–coupled receptor (GPCR) with seven-transmembrane domains (Howard, Feighner et al. 1996).

The human GHS-R1 gene has been identified on chromosome 3, at position q26–27 (McKee, Palyha et al. 1997). The GHS-R1 gene possesses two splice variants, termed as type 1a and type 1b (Gnanapavan, Kola et al. 2002), but only the GHS-R1a isoform responds to ghrelin (Howard, Feighner et al. 1996). GHS-R1a is constituted of 366 amino acids and is expressed with high density in the pituitary and with lower density in regions including the hypothalamus, hippocampus, the ventral tegmental area (VTA), substantia nigra (SN) and dorsal raphe nucleus (DRN) (Guan, Yu et al. 1997). These areas are involved in appetite, cognition, reward, motor control, and anxiety, respectively, indicating that ghrelin may exert regulative effects on those functions through the GSH-R1a.

Ghrelin-mediated functions, through the GSH-R1a, may also be dependent on the availability of certain neurotransmitters. It was shown that ghrelin administration into the VTA led to an increase in the extracellular concentration of dopamine (DA) in the Nucleus Accumbens, a region involved in motivation and reward (Jerlhag, Egecioglu et al. 2007). Another study showed that animals treated with a selective serotonin reuptake inhibitor presented a reduction in ghrelin-induced memory retention (Carlini, Gaydou et al. 2007). These findings suggest that ghrelin effects on memory, reward and possibly on food intake, can depend on the availability of serotonin and DA (Carlini, Gaydou et al. 2007, Jerlhag, Egecioglu et al. 2007). However, it was already reported that GSH-R1a interacts with other receptors. Recently, it was demonstrated that when serotoninergic receptor signaling is blocked, ghrelin's orexigenic effect is potentiated *in vivo*, and that both ghrelin and serotoninergic receptors interact (Schellekens, De Francesco et al. 2015). Another recent study showed that GHS-R1a and the DA receptors type 2 physically interacted with one another and that this interaction altered dopamine signalling, which is known to be involved in feeding behaviors in the hypothalamus (Kern, Albarran-Zeckler et al. 2012). These findings show that ghrelin signalling and ghrelin receptor’s mechanisms and functions are very complex. Not only ghrelin-dependent functions seem to depend on the availability of certain neurotransmitters in the brain, but it also seems that ghrelin receptors may be implicated in the availability of those neurotransmitters. Altogether, GHS-R1a receptors interactions and the signalling of

neurotransmitters such as serotonin and DA appear to be relevant on the ghrelin-mediated functions and should be taken into consideration when studying ghrelin effects in the brain.

Some receptors may exhibit an active conformation, therefore activating signalling pathways, without any need for an agonist (de Ligt, Kourounakis et al. 2000). This phenomenon is known as constitutive receptor activity and can be observed when using an inverse agonist, which is a ligand that blocks the constitutive receptor activity (de Ligt, Kourounakis et al. 2000). The constitutive activity is observed in GHS-R1a due to the presence of 3 aromatic aminoacids located in the 6^{th} and 7^{th} transmembrane helix domains (reviewed in (Holst, Cygankiewicz et al. 2003)). These aminoacids constitute an hydrophobic core that mimics the alteration of conformation induced by GHS-R1a agonists, thereby mimicking agonist activation and stabilizing the receptor in its active conformation (reviewed in (Mear, Enjalbert et al. 2013)). GHS-R1a constitutive activity is very robust and is thought to contribute to GH secretion and body weight regulation but its physiological relevance has not been fully clarified (Mear, Enjalbert et al. 2013).

Despite the fact that GHS-R1a is the only known receptor for ghrelin, both acyl- and deacyl-ghrelin inhibit DNA synthesis and proliferation of prostate cancer cell lines (Cassoni, Ghe et al. 2004). This study indicated that a ghrelin receptor common for both acyl- and deacyl-ghrelin may exist. Another study showed that a ghrelin antagonist inhibits ghrelin-induced GH secretion *in vivo* but acts as an agonist simultaneously as it stimulates weight gain (Halem, Taylor et al. 2005). These observations were accompanied with an increased in Fos- protein immunoreactivity in the dorsal medial hypothalamus (DMH), a region associated with regulation of food intake (Halem, Taylor et al. 2005). These observations indicate that perhaps GHS-R1a may not be the sole receptor for ghrelin and that the mechanisms of action of ghrelin in different tissues may depend on the expression of specific receptor and its properties.

GHRELIN SIGNALLING

GHS-R1a is a GPCR, therefore, it interacts with a heterotrimeric protein complex, composed of three different subunits α, β and γ, of which the α-subunit has a guanine nucleotide binding pocket (reviewed in (Latek, Modzelewska et al. 2012)). At the resting state, the α-subunit (Gα) binds to GDP, but when ghrelin binds to GHS-R1a, the α-subunit shape will change allowing GDP to escape, such that GTP will immediately occupy the free pocket

(Latek, Modzelewska et al. 2012). This leads to a second conformational change, causing the activation of signalling cascades (Latek, Modzelewska et al. 2012). Different Gα subunits engage different effector proteins; therefore leading to the production of different types of second messengers and to the activation of different signalling pathways (Latek, Modzelewska et al. 2012). GHS-R1a binds to different Gα subunits in a cell-specific manner therefore, triggering different signalling cascades (Figure 2).

In pituitary cells, ghrelin binding to GHS-R1a will induce the release of the Gαq (or Gα11) from the dimer Gβγ (Chen, Wu et al. 1996). GTP-bound Gαq subunit will then recruit PLCβ, a phospholipid-cleaving enzyme, to the cell membrane where the phospholipids are. The preferred phospholipid is phosphatidylinositol-4,5-bisphosphate [(PIP_2), reviewed in 38]. PIP_2 will be cleaved by PLCβ into diacylglycerol (DAG), a membrane-bound second messenger, and inositol-1,4,5-trisphosphate (IP_3), a soluble second messenger that diffuses into the cytoplasm. IP_3 will mobilize the Ca^{2+} from intracellular pools such as the endoplasmic resticulum (ER) (Rebecchi and Pentyala 2000). Together with the high DAG levels, this will activate the protein kinase C (PKC), which, in turn, inhibits the K^+ channels and causes cellular depolarization (Rebecchi and Pentyala 2000). The resulting change in voltage will open Ca^{2+} channels leading to Ca^{2+} influx, which will exacerbate the cellular depolarization (Rebecchi and Pentyala 2000). The depolarization in these cells causes GH release (Chen, Wu et al. 1996).

In the hypothalamus, in rat neuropeptide Y-expressing (NPY) neurons, ghrelin induces Ca2+ mobilisation, an effect that was inhibited when researchers used a protein kinase A (PKA) inhibitor (Kohno, Gao et al. 2003). As PKA activation usually results from the activation of another G protein, in this case, Gαs subunit, this study indicated that ghrelin activates the Gαs subunit in the NPY neurons (Kohno, Gao et al. 2003). The Gαs subunit binds and activates the enzyme adenylyl cyclase (AC), which, in turn, converts the ATP into cyclic adenosine monophosphate (cAMP), that will lead to PKA activation (Rebecchi and Pentyala 2000). PKA phosphorylates a variety of proteins involved in functions such as metabolism, gene expression or Ca^{2+} channels activity (Rebecchi and Pentyala 2000). Phosphorylation of Ca^{2+} channels will enable Ca^{2+} efflux, causing neuronal depolarization (Rebecchi and Pentyala 2000). cAMP also activates the cAMP response element binding protein (CREB) which is a well-documented transcription factor involved in the regulation of genes involved in neuronal activation (Rebecchi and Pentyala 2000). Not surprisingly, CREB has been involved

in the regulation of the ghrelin-dependent (and independent) activation of the NPY neurons and of the proopiomelanocortin-expressing (POMC) neurons in the hypothalamus (Shimizu-Albergine, Ippolito et al. 2001, Lage, Vazquez et al. 2010), which play an essential role in the regulation of feeding behavior and energy expenditure.

In the hippocampus, ghrelin administration directly into its ventral subregion increased food intake in rats, an effect that was blocked by the co-administration with a phosphatidylinositol-3-kinase (PI3K) inhibitor (Kanoski, Fortin et al. 2013). Moreover, Ribeiro and co-workers (2014) showed that inhibition of the PI3K or the PKA pathways prevented the ghrelin-induced GluA1 incorporation in the cell membrane (Ribeiro, Catarino et al. 2014), therefore suggesting that Gαq subunits may be coupled to the GSH-R1a in the hippocampus.

Nevertheless, by increasing the intracellular concentration of Ca^{2+}, ghrelin can also exert protective effects against vascular inflammation through the activation of the calmodulin-dependent kinase kinase (CaMKK), AMP- activated protein kinase (AMPK) and indirectly of endothelial nitric oxide synthase [(eNOS), reviewed in (Castaneda, Tong et al. 2010). The activation of AMPK can promote the activation of the acetyl-CoA carboxylase (ACC) and the inhibition of fatty acid synthase (FAS) in the ventral medial hypothalamus induced in the presence of ghrelin, therefore, regulating food intake (Castaneda, Tong et al. 2010).

GSH-R1a can also bind to other Gα subunits, including the $G\alpha_{i/o}$ subunit, which mediates the inhibition of the activation of cAMP (reviewed in (Gao and Horvath 2007)) and the $G\alpha_{12/13}$ subunit which is involved in the regulation of the actin cytoskeletal remodeling (Wang, Tan et al. 2006).

As mentioned above, the GHS-R1a also presents a high degree of constitutive activity and the signalling pathways activated in this case include the PLC- PKC pathway (Mousseaux, Le Gallic et al. 2006). Damian and colleagues observed that the receptor *per se* activates Gα11 in the absence of agonist (Damian, Marie et al. 2012) corroborating the preliminary data supporting that GHS-R1a binds to Gα11 even in the absence of an agonist to activate PLC-PKC pathway. These results suggest a relevant physiological role for the GHS-R1a constitutive activity.

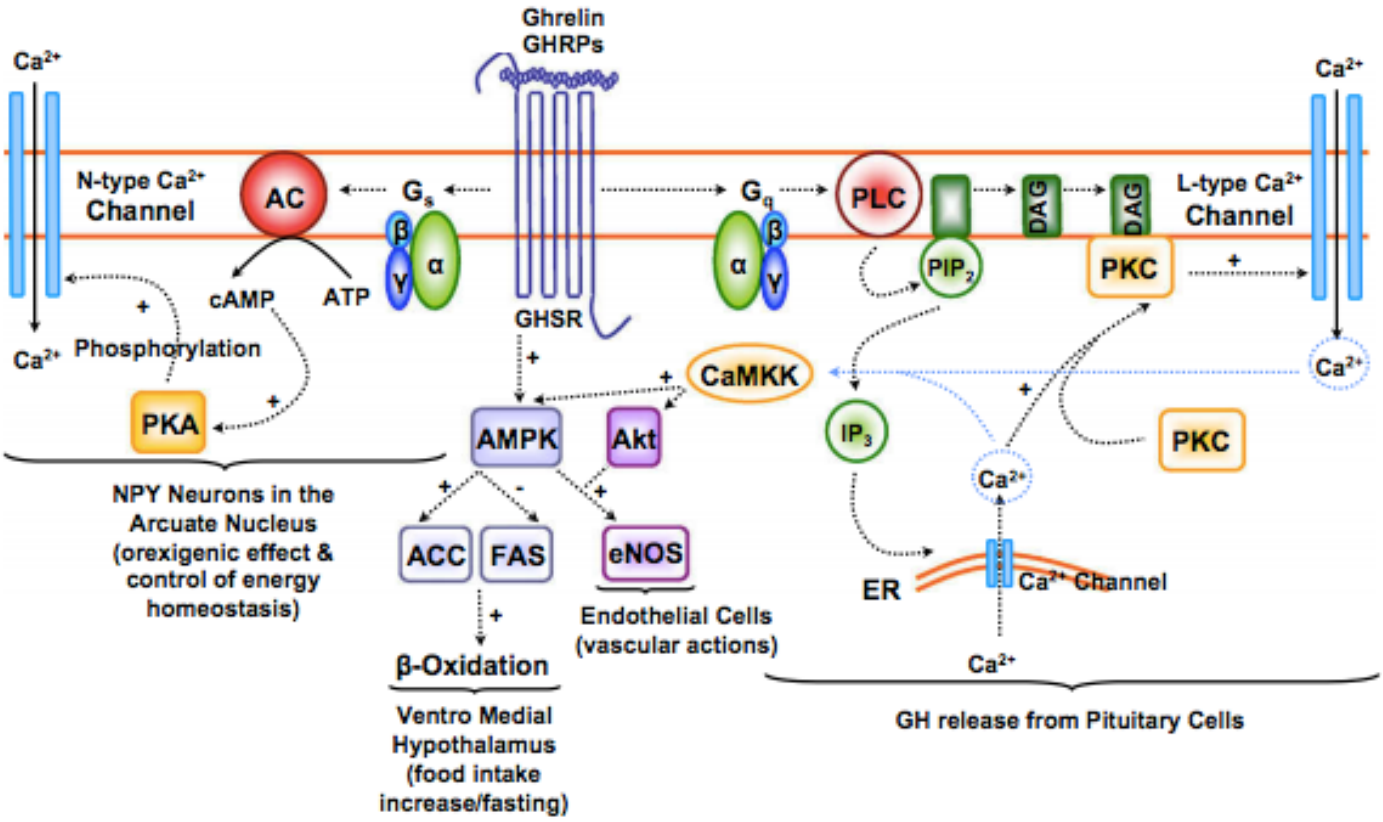


Figure 2. Signal transduction pathways activated by GHS-R1a. Ghrelin can increase intracellular levels of calcium through the AC–PKA pathway in NPY neurons of the ARC or through the PLC–PKC pathway in the pituitary. Ghrelin can also inhibit vascular inflammation through the activation of the CaMKK, AMPK and eNOS. AMPK together with ACC and FAS also mediate ghrelin increase in food intake in relation to fasting. Adapted from (Castaneda, Tong et al. 2010).

BIOLOGICAL FUNCTIONS OF GHRELIN

Ghrelin has been identified in many species. The amino acid sequences of mature ghrelin is well conserved across mammals, including human, rat, mouse, rhesus monkey, mongolian gerbil, cow, pig, sheep and dog, which reinforces the idea of a relevant physiological role for ghrelin (reviewed in (Kojima and Kangawa 2005)). As stated before, ghrelin, the hunger hormone, seems to be involved in the regulation of appetite as well as in higher brain functions. Under an evolutionary point of view, it seems important and even necessary to have a hormone that enables the association of food with spatial localization, for instance. This allows animals to remember where they can find food again, and constitutes a survival strategy. Therefore, it is not surprising that ghrelin is present and well conserved among species. Ghrelin most studied physiological functions are the GH secretion, appetite and energy homeostasis, food reward and cognition. For this reason, these will be the biological functions to be approached in detail.

HORMONE SECRETION

As aforementioned, ghrelin was first described as a GHS. The GH secretion is regulated by the hypothalamus, a brain region responsible for the secretion of many body's hormones which, in turn, regulate the secretion of pituitary hormones. GH is produced in the anterior pituitary, and its secretion is primarily regulated by two hypothalamic peptides: growth-hormone- releasing hormone (GHRH, stimulatory) and somatostatin [(inhibitory), reviewed in (Castaneda, Tong et al. 2010)]. Ghrelin acts at both hypothalamic and pituitary levels to modulate GH secretion as its receptors are expressed in these tissues(Kojima, Hosoda et al. 1999, Popovic, Miljic et al. 2003). In the pituitary, ghrelin binds to GHS-R1a directly on the somatotropic cells to stimulate not only the GH secretion (Popovic, Miljic et al. 2003) but also the secretion of adrenocorticotropic hormone, cortisol, and prolactin (Schmid, Held et al. 2005, Correa-Silva, Nascif et al. 2006). Nevertheless, loss of GHS-R1a constitutive activity leads to impaired GH release and short stature in humans (Pantel, Legendre et al. 2006), indicating that the GHS-R1a constitutive activity *per se* is physiologically involved in GH release.

APPETITE, FOOD INTAKE AND ENERGY HOMEOSTASIS

Peripheral nutrient-sensing molecules, such as leptin and ghrelin, communicate with neuronal networks to regulate energy homeostasis. In the ARC, two types of neurons have opposite effects on feeding. The POMC neurons produce anorectic effects when stimulated by leptin, the "satiety hormone," produced by adipose cells (reviewed in (Gao and Horvath 2007)). On the other hand, the NPY neurons and the agouti gene- related transcript expressing (AgRP) neurons promote feeding when they are activated by the circulating ghrelin that was secreted by the empty stomach in the presence of a negative energetic status [(Gao and Horvath 2007), Figure 3]. Ghrelin binds mostly on presynaptic terminals of NPY and AgRP neurons (Cowley, Smith et al. 2003) facilitating the presynaptic release of NPY, AgRP and of γ-amino butyric acid (GABA). AgRP is an antagonist of the alpha melanocortin (αMSH) receptors MC3 and MC4, inhibiting aMSH from activating its receptor; this will promote appetite (Gao and Horvath 2007). In turn, GABA is an inhibitory neurotransmitter that will suppress the POMC neurons (Cowley, Smith et al. 2003). Altogether, these events will enable a dominant increase in the activity of the neurons that are involved in the stimulation of appetite (NPY neurons) and is therefore involved in the

promotion of food intake (Cowley, Smith et al. 2003). In fact, these neurons are really close to capillaries, therefore facilitating the uptake of several signals that circulate in the blood such as ghrelin (Benoit, Schwartz et al. 2000, Cone 2005).

If ghrelin is involved in food intake, it is not surprising that ghrelin levels might be altered in eating disorders and obesity. In fact, in obese individuals, ghrelin concentrations in blood are reduced compared to lean control subjects (Tschop, Weyer et al. 2001), but whether this is cause or effect is not defined. In turn, in patients with anorexia nervosa, ghrelin concentrations in blood are higher than normal plasma ghrelin levels, which decrease if weight gain occurs(Soriano-Guillen, Barrios et al. 2004). These findings offer hope for clinical perspectives of ghrelin analogues acting as agonists or antagonists for treatment of eating disorders in the future.

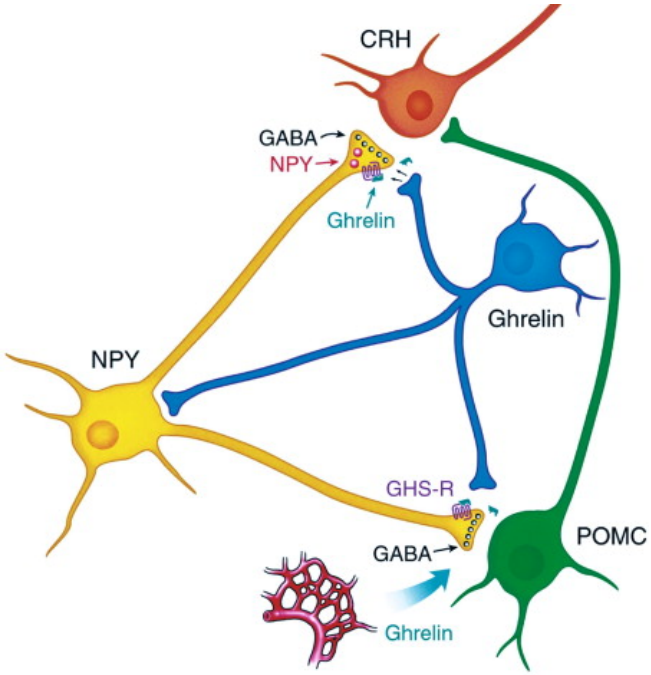


Figure 3. Ghrelin effects on hypothalamic circuits. The axons of hypothalamic ghrelin neurons abut NPY axons presynaptically in the ARH and in the PVH. Ghrelin binds to GHS-R1a to increase the release of GABA, NPY and AGRP from the terminals of NPY axons. GABA and neuropeptide secretion modulates the activity of postsynaptic POMC and corticotropin-releasing hormone (CRH) neurons. Adapted from (Cowley, Smith et al. 2003).

FOOD REWARD BEHAVIOR

Metabolic need (negative energy balance) is not the only motivation to consume food (reviewed in (Grill, Skibicka et al. 2007)). Other motivations include available attractive foods, temporal factors (time of day, season), emotion, and cognition (Grill, Skibicka et al. 2007). Collectively, these factors influence "nonhomeostatic" food intake (Grill, Skibicka et al. 2007). Ghrelin is not only involved in the research of food for the digestive function but also for reward, by acting on hindbrain and midbrain areas, respectively (Jobst, Enriori et al. 2004). On the midbrain, the reward pathway, also known as the mesolimbic pathway, is a dopaminergic pathway that is involved in motivation, cognition and reward and integrates mainly the VTA and the NA (reviewed in (Kenny 2011)). High levels of the GHS-R1a have been reported in the VTA (Zigman, Jones et al. 2006) and the direct ghrelin administration in the VTA induced overflow of dopamine within the NA as measured by microdialysis in freely moving mice (Jerlhag, Egecioglu et al. 2007), which supports a role for ghrelin in the VTA-mediated reward signaling. Moreover, ghrelin injection in VTA stimulates the intake of palatable food (Egecioglu, Jerlhag et al. 2010). These data indicate that ghrelin plays an important role, not only in food intake, but also in food reward behaviour through the mesolimbic pathway. These ghrelin effects can also contribute to obesity and other related disorders as food rewarding effects can underlie excessive and uncontrolled food intake. Thus, understanding the mechanisms of action of ghrelin in the VTA and NA and other potential analogues can offer potential avenues to treat such conditions.

MEMORY AND ANXIETY

Boosting memory during food searches can help animals to remember where the food can be found next time they are hungry, which is particularly important when food is scarce. The ability of remembering where food can be found achieved in part by learned associations with stimuli that predict nutrient availability (reviewed in (Sclafani 1997)). Hippocampus, amygdala and DRN are the main brain areas involved in learning and memory mechanisms, therefore one can speculate their involvement in memories associated with food intake. Regarding memory function, it was shown that injections of ghrelin in the hippocampus and DRN increased food intake and memory performance in a dose-dependent manner (Carlini, Monzon et al. 2002). A recent study corroborates these observations (Li, Chung et al. 2013). In this study, memory was impaired in ghrelin knockout mice; this correlated with a reduction in the number of progenitor cells in the subgranular zone (SGZ), a reduction in the fraction of

immature neurons and a reduction of newly generated neurons (Li, Chung et al. 2013). Another study showed that ghrelin receptor knockout mice present spatial memory impairments (Davis, Choi et al. 2011). Diano and co-workers (Diano, Farr et al. 2006) also showed that peripheral ghrelin increases spine density and synaptic organization in the hippocampus, which correlated with improvements in memory performance. Altogether, this suggests that a gut-hippocampus axis may facilitate memory retention for the spatial localization of food (Moran and Gao 2006) and implies that ghrelin exerts cognitive functions and can also be thought as a potential cognitive enhancer. Yet so, the mechanisms involved in this processes remain unknown.

Of note, in the regard of anxiety, the role for ghrelin remains even less clear. While in one study, ghrelin was shown to increase anxiety (Carlini, Monzon et al. 2002), in another, ghrelin was shown to reduce anxiety after acute stress when the hypothalamic-pituitary-adrenal axis was stimulated at the level of the anterior pituitary (Spencer, Xu et al. 2012).

HIPPOCAMPUS

The hippocampus is a crucial structure for the formation of certain types of memory, such as episodic, declarative and spatial memory (Wang and Morris 2010). It is composed of three main regions: the CA1 and the CA3 regions and the dentate gyrus [reviewed in (Kullmann and Lamsa 2007), Figure 4]. The main inputs the hippocampus receive come from the perforant pathway from the entorhinal cortex [cortex surrounding the hippocampus, (Kullmann and Lamsa 2007)]. These axons connect with the granule cells of the dentate gyrus, which sends axons (mossy fibres) that will establish synapses with the pyramid excitatory cells in the CA3 (Kullmann and Lamsa 2007). The axons of the CA3 pyramidal cells region, or the Schaffer collaterals project to the CA1 layers where they will do synapses (Kullmann and Lamsa 2007) and to other regions of the brain. As mentioned above, GSH-R1a is expressed in the hippocampus and ghrelin deficient mice were already shown to present impairments in hippocampal-dependent functions (Diano, Farr et al. 2006), suggesting a role for ghrelin in those functions.

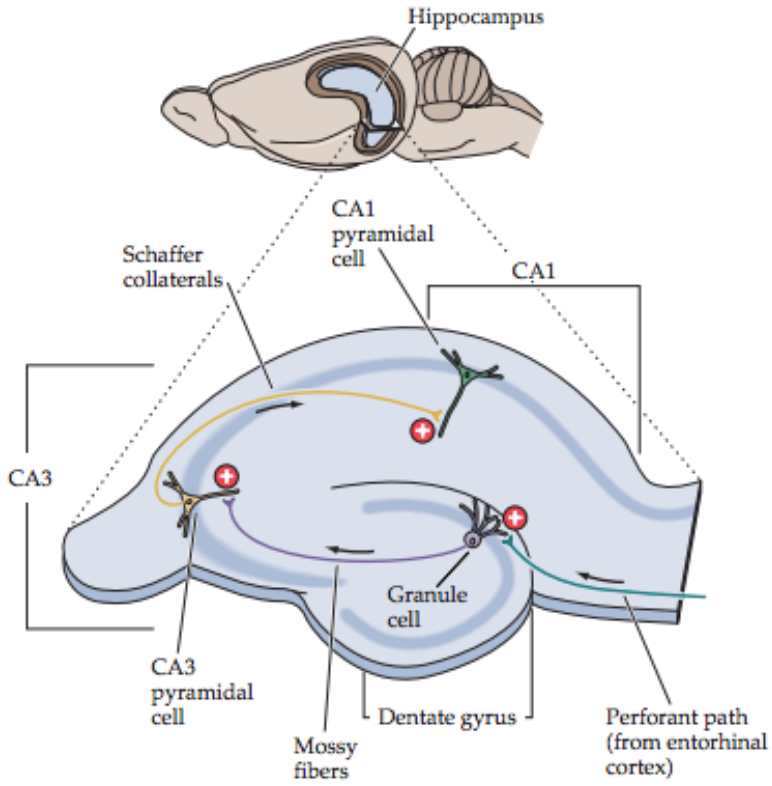


Figure 4. Anatomy of the rodent hippocampus showing the major regions, excitatory pathways and synaptic connections. Adapted from (Purves 2001).

GLUTAMATERGIC SYNAPSES

Neurons communicate through synapses, which can be inhibitory when they promote the hyperpolarization of the postsynaptic cell or excitatory when they do the opposite. The majority of the excitatory synapses in the brain involve glutamate as neurotransmitter (reviewed in (Chua, Kindler et al. 2010)), and these synapses are termed glutamatergic synapses, Figure 5). As glutamatergic synapses are very abundant in the hippocampus, its functional role on hippocampal-dependent functions is robust and has been extensively studied (Chua, Kindler et al. 2010). It was shown that a large fraction of GHS-R1a localizes to glutamatergic synapses in the hippocampus (Ribeiro, Catarino et al. 2014), highlighting the potential relevance for ghrelin in hippocampal-dependent functions.

GLUTAMATE RECEPTORS

Glutamatergic transmission is possible because glutamate, which is released from presynaptic vesicles into the synaptic cleft, activates specific receptors at the postsynaptic cell (Chua, Kindler et al. 2010), the glutamate receptors. These receptors are clustered at the postsynaptic membrane in a rich protein network that includes anchoring and scaffolding proteins such as PSD-95; signalling proteins such as CamKII and cytoskeletal elements called the postsynaptic density (PSD) (reviewed in (Sheng and Kim 2011)).

Glutamate receptors may be ionotropic or metabotropic. Both types of receptors are composed of different subunits, which exhibit distinct biophysical properties, therefore those subunits mediate distinct physiological roles critical for synaptic function (reviewed in (Hollmann, Boulter et al. 1994)). In one hand, metabotropic glutamate receptors indirectly activate ion channels on the plasma membrane through signalling cascades that involve the activation of G proteins. In turn. ionotropic glutamate receptors are themselves an ion channel pore that assembles in that conformation following glutamate binding. Ionotropic glutamate receptors include alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic-acid receptors (AMPARs), Kainate receptors, N-methyl-D-aspartate receptors (NMDARs) and delta receptors (reviewed in (Smart and Paoletti 2012)). As the ionotropic receptors are the most studied and understood concerning synaptic transmission and its properties, these will be the ones approached in more detail below.

NMDARs trigger the synaptic incorporation of AMPARs and are highly expressed in the hippocampus where they are crucial for the mechanisms of learning and memory (reviewed in (Paoletti, Bellone et al. 2013)). NMDARs form tetramers that can be composed of GluN1-GluN3 subunits (Paoletti, Bellone et al. 2013). They are blocked by extracellular Mg^{2+} ions, being activated by glutamate and glycine together with an increase in voltage (reviewed in (Dingledine, Borges et al. 1999)). The change in voltage due to the increase of concentration of cations inside the cell will displace the Mg^{2+} from the channel pore, therefore, allowing the influx of Na^{+} and the influx of small amounts of Ca^{2+} ions exacerbating cell depolarization (reviewed in (Dingledine, Borges et al. 1999)).

AMPARs mediate fast excitatory transmission and they are also implicated in memory formation (Dingledine, Borges et al. 1999). There are four AMPARs subunits, GluA1-4 which can assemble into several possible combinations to form AMPARs (Dingledine, Borges et al. 1999). Following glutamate binding, AMPARs will open and become permeable to Na^{+} and K^{+} ions, resulting in cell depolarization (Dingledine, Borges et al. 1999). At mature

hippocampal excitatory synapses, AMPARs consist mainly of GluA1/GluA2 and, in some cases, of GluA2/GluA3 subunits (Wenthold, Petralia et al. 1996). GluA2 subunit has specific properties. GluA2 mRNA has an editing site where the uncharged amino acid glutamine (Q) is replaced by the positively charged arginine (R) in the receptor's ion channel (Burnashev, Monyer et al. 1992). This will render the channel impermeable to calcium (Burnashev, Monyer et al. 1992).

Ghrelin was shown to trigger the synaptic incorporation of the GluA1 subunit of the AMPARs in hippocampal neurons (Ribeiro, Catarino et al. 2014) suggesting that the ghrelin effects on memory may depend AMPARs trafficking to the synapse.

DENDRITIC SPINES

Glutamatergic synapses occur on short protrusions joined to the main dendrite by a thin neck called dendritic spines (reviewed in (Bhatt, Zhang et al. 2009), Figure 5). These structures, which appear early during development and increase the surface area of dendrites, are more abundant in higher brain regions such as the hippocampus (Bhatt, Zhang et al. 2009), indicating a relevant physiological role for dendritic spines in the hippocampus.

Dendritic spines present different morphologies. In the hippocampus, most spines have constricted necks and are either mushroom shaped with heads exceeding 0.6 microns in diameter or thin shaped with smaller heads (Harris, Jensen et al. 1992). Dendritic spines also present other shapes termed as stubby or branched. In stubby spines, the head widths equal to neck lengths while branched spines have two or more heads (Bourne and Harris 2008). In turn, the dendritic filopodia are long and thin protrusions without bulbous heads (Bourne and Harris 2008), see figure 6.

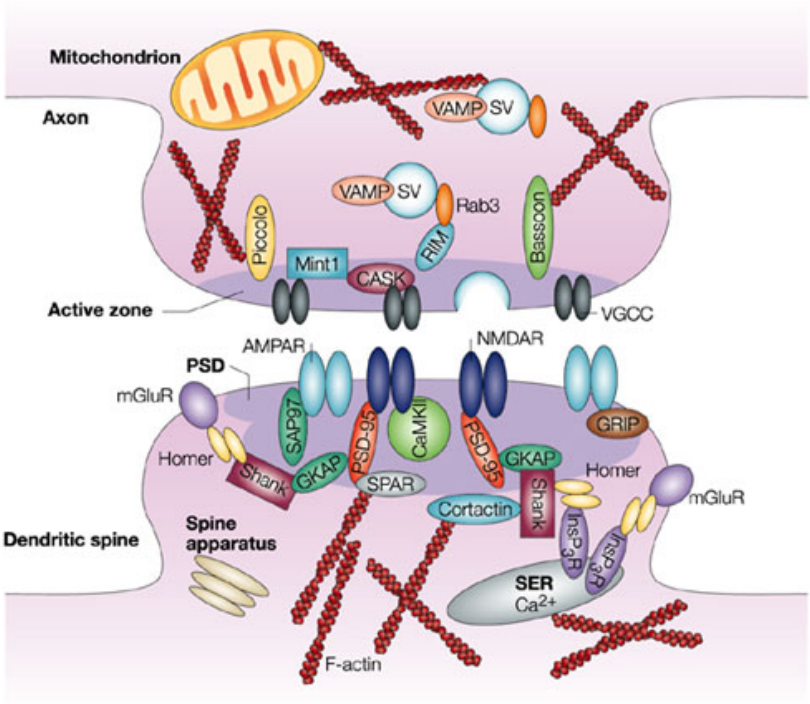


Figure 5. **Molecular composition and structure of a glutamatergic synapse.** Synaptic vesicles (SVs) on the presynaptic terminal are loaded with glutamate and carry proteins such as VAMP (vesicle-associated membrane protein). Upon despolarization of the postsynaptic neurons, voltage-gated calcium channels (VGCCs) trigger fusion of SVs with the plasma membrane, which leads to neurotransmitter release. Vesicle fusion occurs at the active zone, which is characterized by specific proteins such as Bassoon and Piccolo. The active zone is directly opposed to the PSD, which contains a high concentration of glutamate receptors — NMDARs and AMPARs — and their associated scaffold proteins (for example, PSD-95, SAP97 (synapse-associated protein), GRIP (glutamate-receptor-interacting protein), as well as signalling molecules, such as SPAR (spine-associated Rap GTPase-activating protein) and CaMKII. Metabotropic glutamate receptors (mGluRs) are shown lying outside the PSD. Adapted from (Li and Sheng 2003).

The size, or volume, and shape of spines correlates with their properties (Bhatt, Zhang et al. 2009). Larger spines are functionally stronger and stable. Stronger because they have more space to accommodate receptors in the membrane and other functionally important molecules that will regulate intracellular calcium, endosomal recycling, protein translation and degradation, and interaction with astroglia, therefore, increasing the synaptic strength (Bhatt, Zhang et al. 2009). And stable because they do not suffer changes in shape and volume as often or do not disappear easily (Bhatt, Zhang et al. 2009). The mushroom spines are an example of these larger spines and have been correlated with memory persistence (reviewed (Bourne and Harris 2008)). In turn, smaller spines may be more flexible, rapidly enlarging or shrinking in response to subsequent activation (Bhatt, Zhang et al. 2009). These spines are specifically important during learning processes. Dendritic filopodia are not spines but these structures share properties with the smaller spines and can be, therefore, used as an example of smaller spines. Filopodia act as spines precursors because they can easily

become a spine, therefore, they are thought to be the substrate for learning and memory processes (Bourne and Harris 2008).

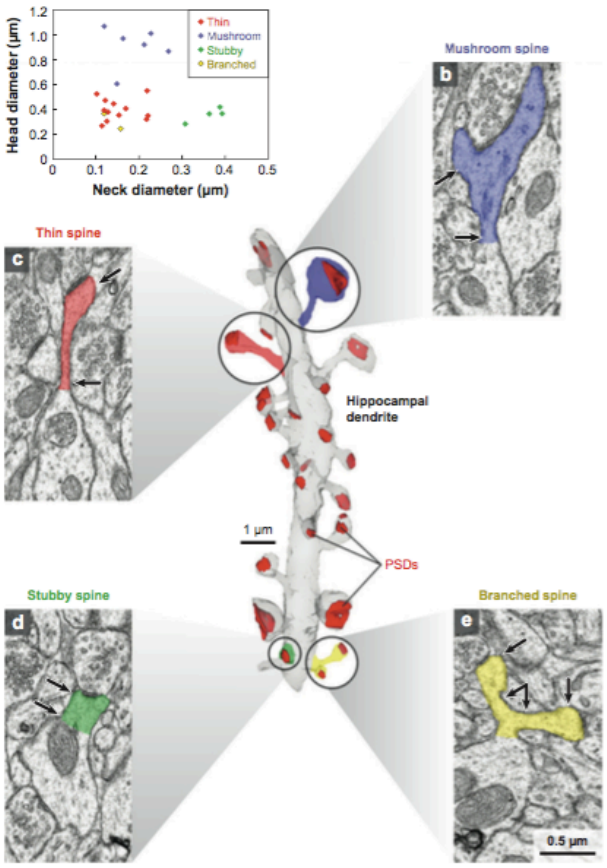


Figure 6. Dendritic spines' shape and size. A three-dimensional reconstruction of a hippocampal dendrite (gray) illustrating different spine shapes including mushroom (blue), thin (red), stubby (green), and branched (yellow). PSDs (red) also vary in size and shape. (a) A graph plotting the ratio of head diameters to neck diameters for the spines on the reconstructed dendrite. Adapted from (Bourne and Harris 2008).

NEUROPLASTICITY

The brain can be thought as a dynamic system therefore it is susceptible of changing. The experience- or activity-dependent changes that occur in the brain reflect its capacity to process and integrate new information and are thought to be the basis for learning and memory formation. This capacity of the brain to be plastic gave rise to the concept of neuroplasticity (reviewed in (Caroni, Donato et al. 2012)). Neuroplasticity involves, therefore, changes in all brain structures at the molecular, cellular and structural level, therefore, is usually classified in two main types: synaptic and structural plasticity (Caroni, Donato et al. 2012).

SYNAPTIC PLASTICITY

Synaptic plasticity is the ability of synapses to strengthen or weaken over time, in response to increases or decreases in neuronal activity (reviewed in (Citri and Malenka 2008)). Many forms and mechanisms of synaptic plasticity have been described but usually they involve the phosphorylation of AMPARs which regulates channel localization, conductance, and open probability and the increase of number of AMPARs and NMDARs at the synapse whether it is due to incorporation from intracellular pools or due to lateral mobilisation from extra-synaptic sites (Citri and Malenka 2008). One form of synaptic plasticity can last on the order of milliseconds to several minutes. This form of synaptic plasticity, known as short-term synaptic plasticity, induces a temporary modification in synaptic efficacy, such that without continued presynaptic activity, the synaptic efficacy will quickly return to its baseline level (Citri and Malenka 2008). On the contrary, if the presynaptic activity is persistent, the synaptic strength will suffer modifications that will persist for minutes, hours or more, a phenomena that is called long-term synaptic plasticity and is thought to underlie the formation of long-lasting memories (Citri and Malenka 2008).

STRUCTURAL PLASTICITY

The tight relationship between structure and function implies that synaptic plasticity results in concomitant structural changes, which under a mechanical point of view makes sense because the molecules that increase the synaptic strength such as signalling molecules, PSDs, AMPARs and NMDARs require more space and a bigger surface area (reviewed in (Kasai, Fukuda et al. 2010)) to be accommodated, when they are recruited to spines, upon an increase in neuronal activity. Indeed, a recent study showed that PSD size correlates with spine size (Meyer, Bonhoeffer et al. 2014). Simply, an increase in the volume and surface area of the spine positively correlates with an increase in synaptic strength. Indeed, activity-dependent synaptic changes are paralleled with structural alterations, which overall contribute for the expression of functional plasticity (Kasai, Fukuda et al. 2010) and may correlate with memory and learning processes. These activity-dependent structural alterations are called structural plasticity (Kasai, Fukuda et al. 2010).

SPINE STRUCTURAL PLASTICITY

Dendritic spines, the structural recipients for glutamatergic synapses, are highly dynamic. For example, Yasumatsu and colleagues showed that, when NMDA receptors and Na^+ channels were completely blocked to prevent activity-dependent plasticity, spines still underwent volume alterations (Yasumatsu, Matsuzaki et al. 2008), showing that spines are really dynamic structures as they suffer changes spontaneously.

However, the morphology of spines undergoes changes whether it occurs spontaneously or in an activity-dependent manner. Spine turnover, which includes both loss of existing spines and gain of new ones, affects a small subpopulation of spines under conditions of baseline activity (Caroni, Donato et al. 2012). Though, under conditions of behavioural learning or when an increase in neuronal activity is induced, changes in spine turnover and spine morphology are more robust and spread and positively correlate for remodelling of neuronal connections and circuitry, which may reflect alterations in memory and learning processes ((Caroni, Donato et al. 2012) Figure 7). These activity-dependent structural changes at the level of spines are called spine structural plasticity. Altogether, one way of assessing spine dynamics, whether under baseline or increased activity conditions is by investigating the changes in shape, volume and number of spines.

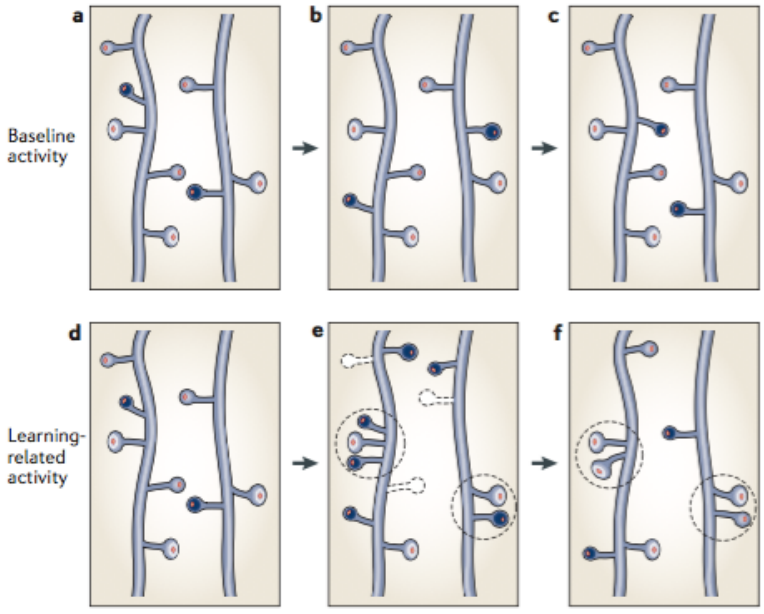


Figure 7. Structural changes in spines under different conditions of activity. Schematic showing a characteristic spine turnover sequence a-c) under baseline activity conditions, the transient spines are represented as the small dark spines. d–f) Under conditions of behavioural learning, this turnover is markedly enhanced, leading to the formation of additional new spines (small dark spines), and the elimination of pre-existing spines (dashed spines). The new spines formed following learning tend to occur in clusters (encircled areas) and exhibit a higher probability to become stabilized as persistent spines, introducing a lasting modification of the synaptic network. Adapted from (Caroni, Donato et al. 2012).

LONG-TERM PONTENTIATION (LTP)

Although several different forms of long-term synaptic plasticity exist, the best-known and the most studied examples of activity-dependent long-term synaptic plasticity are LTP and long-term depression (LTD) of synapses (Citri and Malenka 2008). LTP corresponds to a persistent increase in the synaptic strength in response to an increase in activity induced by a long patterned stimulus or chemically, while LTD is the long lasting decrease in synaptic strength (Citri and Malenka 2008). As functional correlates with structural plasticity, it would be expected that LTP could also be involved in spine structural plasticity. Among several other studies, this was observed by Matsuzaki and co-workers, as LTP easily induced the enlargement of small spines (Matsuzaki, Honkura et al. 2004). This type of studies reinforces the relevance of spines in memory and learning processes. If in one hand, new spines are thought to be the seed of new memory (Caroni, Donato et al. 2012). In another, enlarged spines may help to explain the long-term maintenance of LTP, given that the number of functional AMPARs correlates with spine volume (Matsuzaki, Ellis-Davies et al. 2001, Tsutsui and Oka 2001) which correlates with spine strength, and this, in turn, correlates with memory improvement as referred above. Also above, it was mentioned that GHS-R1a triggers the synaptic incorporation of AMPARs (Ribeiro, Catarino et al. 2014). Given the functional role of AMPARs in the induction of LTP, the same authors were interested in understanding if ghrelin could enhance LTP. This was observed; ghrelin receptor activation was shown to enhance LTP of the synapses between Schaffer collateral fibers and CA1 pyramidal cells in the hippocampus (Ribeiro, Catarino et al. 2014).

MOLECULAR MECHANISMS OF SPINE DYNAMICS

The molecular mechanisms that govern changes in shape, volume and number of spines, also called as spine dynamics, are usually common between conditions of baseline activity or during LTP. These mechanisms involve the participation of several molecules which are implicated in the synapse and spine formation and include adhesion molecules, PSD proteins, protein kinases and phosphatases and actin cytoskeleton and its regulatory elements (Bhatt, Zhang et al. 2009). In particular, the actin cytoskeleton is crucial for spine remodelling. Therefore, regulators of the actin cytoskeleton, mainly actin-binding proteins will be, as well, relevant in the dynamic of dendritic spines.

PI3K and PKA pathways already were described to be involved in spine dynamics (Ribeiro, Catarino et al. 2014). Therefore, not unpredictably, these pathways have also been involved in actin remodelling. In fact, PKA was shown to phosphorylate directly LIM domain kinases (Limk1), an enzyme that is indirectly involved in the regulation of the actin cytoskeleton (Nadella, Saji et al. 2009). Another study used pharmacological approaches to show that PI3K activity alone is sufficient to remodel actin filaments (Qian, Corum et al. 2004). Nevertheless, it was already shown that PI3K activity promotes the dephosphorylation of cofilin, another protein which role is crutial in actin filament dynamics (Nishita, Wang et al. 2004). Indeed, LIMK-1 participates in actin cytoskeletal reorganization, promoting phosphorylation of cofilin (Yang, Higuchi et al. 1998). In 2013 a study finally revealed the link between LIMK–cofilin pathway and spine formation as the authors showed that the activation of this pathway led to a rapid spine formation in response to the pulsatile release of a certain group of hormones (Liston, Cichon et al. 2013), a property that is shared with ghrelin (bearing in mind its patterns of release - before meals).

ACTIN CYTOSKELETON IN SPINE DYNAMICS

Changes in spines size, number and shape are typically dependent on the dynamics of the actin cytoskeleton, which is mainly composed of actin filaments – polymerized actin (F-actin) (Korobova and Svitkina 2010). As stated before, these changes occur spontaneously under baseline activity conditions but also when neuronal activity is increased. Concerning the last, Fukazawa and collaborators showed that LTP induction in the dentate gyrus of rats increases the content of F-actin in spines in the hippocampus, and these elevated F-actin levels persisted for at least five weeks (Fukazawa, Saitoh et al. 2003). The PSD and F-actin content within spines induced by LTP was also correlated with an increase in the spine-size, which makes sense as PSD allows the incorporation of the AMPARs at the synapse and the F-actin enables the enlargement of spines . Indeed, preliminary data from our lab are consistent with these studies as we showed that the ghrelin-induced increase in PSD-95 and F-actin in hippocampal neurons correlates with an increase in the dendritic filopodia (Fiona van Lewmeen, 2013). Moreover, as filopodia are thought to be the substrate of learning, these results indicate that ghrelin may be involved in hippocampal-dependent learning processes.

Regarding the sequence of events that leads to the alterations in spine morphology and density, briefly, as LTP is induced, the F-actin at the spine heads is initially depolymerized

but maintenance of LTP and sustained spine enlargement require polymerization of F-actin (Fukazawa, Saitoh et al. 2003, Bourne and Harris 2008). As stated before, the molecular regulators of F-actin as well as their interacting proteins regulate changes in the density of dendritic filopodia and spines *in vivo* and *in vitro* (Bhatt, Zhang et al. 2009). LIMK1 and Cofilin are just two examples of regulators of F-actin, therefore, modulators of dendritic spines' dynamics, but there are many others (Table 1). Cofilin, which is inactivated by phosphorylation at Ser-3 by LIMK1, plays an essential role in F-actin dynamics (Meng, Zhang et al. 2002). LTP or its learning-induced phosphorylation decreases its affinity for actin, promoting polymerization of the actin filaments and promoting spine enlargement (Fedulov, Rex et al. 2007). So far, it seems that there are no published works reporting the effect of ghrelin on actin dynamics through cofilin, but given the data available, this is an attractive hypothesis.

Table 1. Molecular mediators of spine morphology. Adapted from (Bourne and Harris 2008)

Protein	Function	Protein	Function
PSD-95	Stabilizes nascent spines and anchors receptors and scaffolding proteins at the synapse.	**CamKII**	Increases the thickness of the PSD and phosphorylates signaling molecules involved in plasticity.
Actin	Regulates the extension of filopodia and mediates the expansion of spine heads with LTP and the shrinkage of spine heads with LTD.	**Profilin**	Promotes activity-dependent actin polymerization and stabilizes actin.
Cofilin	Depolymerizes actin filaments, but LTP or learning-induced phosphorylation decreases its affinity for actin, promoting polymerization and spine enlargement.	**Rap1/AF-6**	Elongates spines and removes AMPA receptors with activation, whereas inactivation enlarges spines and recruits AMPA receptors.
Myosin IIb	Stabilizes mushroom spines.	**Myosin VI**	Regulates clathrin-mediated endocytosis of AMPA receptors.
Synaptopodin	Binds to the spine apparatus and may mediate interactions between the actin cytoskeleton and calcium signaling	**Telencephalin**	Slows the development of dendritic spines with overexpression, whereas deletion accelerates the spine development. May be involved in maintaining filopodia during development.
SynGAP	Maintains filopodia during development and localizes to the synapse.	**miR-134**	Negatively regulates spine development by inhibiting translation of Limk1.
N-cadherin	Stabilizes mature synapses and regulates spine morphology and synaptic efficacy.	**EphrinB**	Clusters receptors and mediates spine morphology by recruiting molecules involved in actin polymerization.

OBJECTIVES OF THE PRESENT STUDY

Previous work from our lab showed that:

a) GHS-R1a activation induces an increase in the number of clusters of PSD-95 co-localized with vGlut1 (post- and pre-synaptic markers, respectively, of excitatory synapses) and of F-actin, in cultured hippocampal neurons (Ribeiro, Catarino et al. 2014) Joni van Leeuwen Master's thesis, 2013);
b) GHS-R1a activation induces activity-dependent GluA1-AMPAR synaptic delivery in CA1 hippocampal neurons and enhances NMDAR-dependent LTP in the hippocampus (Ribeiro, Catarino et al. 2014).

There is further evidence in the literature indicating that GHS-R1a activation impacts hippocampal spine density (Diano, Farr et al. 2006); however, these studies are preliminary, and the role of GHS-R1a activation in hippocampal spine dynamics has never been addressed. Therefore, the objectives of this thesis project were to:

1) Establish an imaging approach to study dendritic spine morphology, density, head diameter and head volume in organotypic hippocampal slices;
2) Optimize a time-lapse imaging protocol to follow individual spines through time in organotypic hippocampal slices;
3) Assess the effects of GHS-R1a activation in hippocampal spine dynamics, in two different paradigms that mimic the physiological status of ghrelin: long-term stimulation during 20h (to mimic fasting) and short-term stimulation for 50min (to mimic the peak in ghrelin associated with meals);
4) Explore the signalling pathways activated upon GHS-R1a activation in the hippocampus, in particular the effects on the phosphorylation of cofilin, given the role of cofilin in the regulation of cytoskeletal changes associated with spine dynamics.

Chapter 2
MATERIAL AND METHODS

MATERIALS

GHS-R1a agonist (MK-0677) was purchased from Axon Medchem (Groningen, The Netherlands) and reconstituted in PBS (pH 7.4). Tetrodotoxin (TTX) was purchased from Tocris Bioscience (Bristol,UK).

Table 2. Primary antibodies used in the immunocytochemistry and western blot experiments

Primary Antibodies	Dilution (application)	Source
Anti-β-Actin mouse	1:5000 (WB)	Sigma-Aldrich (Sintra, Portugal)
Anti-MAP2 chicken	1:5000 (ICC)	Abcam (Cambridge, UK)
Anti-vGlut1 guinea pig	1:5000 (ICC)	Millipore (Madrid, Spain)
Anti- S3A phosphorylated-Cofilin (P-cofilin) Rabbit	1:100 (ICC) and 1:1000 (WB)	Cell Signaling Technology (Danvers, USA)
Anti-Total Cofilin	1:10000 (WB)	Abcam (Cambridge, UK)

Table 3. Secondary antibodies used in the immunocytochemistry and western blot experiments

Secondary antibodies	Dilution (application)	Source
Alexa 488-conjugated anti-rabbit	1:500 (ICC)	Millipore Molecular probes
Alexa 647-conjugated anti-guinea pig	1:500 (ICC)	Molecular Probes (Leiden, The Netherlands)
AMCA-conjugated anti-chicken	1:200 (ICC)	Jackson ImmunoResearch (West Grove, USA)
Alkaline phosphatase conjugated anti-mouse	1:5000 (WB)	GE Heathcare (Carnaxide, Portugal)
Alkaline phosphatase conjugated anti-rabbit	1:10000 (WB)	GE Heathcare (Carnaxide, Portugal)

METHODS

ISOLATION PROTOCOLS

HIPPOCAMPAL BANKER NEURONAL CULTURE

Hippocampi from E18-E19 Wistar rat embryos were isolated and dissociated in Ca^{2+} - and Mg^{2+}-free Hank's Balanced Solution (HBSS; 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4 \cdot 2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% (w/v) phenol red) on ice. Hippocampi cells were incubated with 0.06% (w/v) trypsin at 37ºC during 15 minutes. The supernatant was discarded and cells were washed six times in HBSS to remove trypsin, after which they were mechanically dissociated. A mixture of 50µl of Tryptan Blue with 50µl of HBSS and 50µl of the cellular suspension was used to determine the cell concentration. Cells were then plated in 60 mm petri dishes coated with poly-D-lysine (0.1 mg/ml) at a final concentration of 300000 cells/4ml in plating media (Minimum Essential Medium (MEM; GIBCO, Invitrogen, Barcelone, Spain) supplemented with 10% (v/v) horse serum (GIBCO, Invitrogen, Barcelone, Spain), 0.6% (w/v) glucose, and 1mM pyruvic acid) for 2 hours at 37ºC in a humidified incubator of 5% CO_2/95% air. After that period, coverslips were flipped over on astroglial feeder layer in culture dishes containing neuronal culture medium with glutamate. These neurons grew face-down over the feeder

layer but were kept separate from the glia by wax dots on the neuronal side of the coverslips. To prevent the outgrowth of glia, cytosine arabinoside (5 µM) was added to the neuronal cultures after 2-5 days of the isolation. Cultures were maintained in a humidified incubator of 5% CO_2/95% air at 37°C. The cells were fed by replacing one-third of the conditioned medium per dish with fresh neuronal culture medium without glutamate, once every week. Cultures were used after DIV19.

HIPPOCAMPAL ORGANOTYPICAL SLICE CULTURES

Hippocampi from P6-P7 Wistar pups were isolated and dissected on ice cold dissection solution (in mM: 10 glucose, 4 KCl, 24 $NaHCO_3$, 234 sucrose, 0.5 $MgCl_2 \cdot 6H_2O$, 0.7 $CaCl_2 \cdot 2H_2O$, 0.03 phenol red, at pH 7.4) gassed in 5% CO_2/95% air. Hippocampi were then cut in 250 or 300µm thick slices which were then placed onto a slice culture insert (Millipore, Madrid, Spain) inside a well filled with 800-900 µL of culture medium [Minimum Essential Media (MEM; GIBCO, Invitrogen supplemented with 20% (v/v) horse serum (GIBCO, Invitrogen, Barcelone, Spain), 1 mM glutamine, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mg/l insulin, 0.0012% (w/v) ascorbic acid, 30 mM HEPES, 13 mM glucose, 5.2 mM $NaHCO_3$, at pH 7.25-7.26), and final osmolarity of 310-320 mOsm/l]. Cultures were maintained in a humidified incubator of 5% CO_2/95% air at 35°C (Gahwiler et al., 1997). The culture medium was replaced once every two days. Slices were subsequently used for biochemistry or for live imaging experiments after transfection.

TRANSFECTIONS

HIPPOCAMPAL BANKER NEURONAL CULTURE

In order to fill the cells with a fluorescent protein, in both types of cultures, cells were transfected, as described below, with the plentilox 3.7 vector encoding *mCherry* under the control of the synapsin promoter (kind gift from Ann Marie Craig, University of British Columbia, Vancouver). Hippocampal neurons were transfected at DIV11, using the calcium phosphate method adapted from Jiang et al., 2004. Briefly, the coverslips were transferred to a MultiWell 12 plate containing conditioned medium and 2 mM of kyanurenic acid (to decrease the toxicity of the protocol). The DNA was diluted in Tris-EDTA transfection buffer (10 mM Tris-HCl and 2.5 mM EDTA, pH 7.3) to reach the final amount of 2.5µg per coverslip.

$CaCl_2$ solution (2.5 M in 10 mM HEPES) was added, dropwise, to reach a final concentration of 250 mM. This mixture was then added, dropwise, to a HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, 42 mM HEPES, pH 7) of the same volume. The tube containing the mixture was vortexed briefly and a final volume of 62.5 µl of the DNA precipitates was then added, dropwise, to each coverslip. The neurons were incubated with precipitates for 2 hours at 37ºC in a humidified incubator (5% CO_2/95% air). The transfection was stopped by replacing the medium of each well with fresh neurobasal medium (GIBCO, Invitrogen, Barcelone, Spain) without glutamate containing 2 mM of kynurenic acid, acidified with HCl (~5 mM final concentration) for 15-20min in a humidified incubator of 5% CO_2/95% air. Coverslips were then transferred to the original petri dish, and put back in the incubator to allow expression of the transfected construct.

HIPPOCAMPAL ORGANOTYPICAL SLICE CULTURES

Hippocampal organotypical slices were transfected using biolistic transfection (adapted from (O'Brien and Lummis 2006)), according to which hippocampal slices are bombarded with high velocity DNA coated particles. This transfection method is a useful mean for fluorescent labelling of a small subset of neurons in the slice, enabling complete neurons to be seen isolated.

Preparation of microcarriers

Approximately 10 mg of 1.0 µm diameter gold microcarriers were weighted to a microtube, and 50 µl spermidine (0,05 M) was added to the gold followed by the addition of an equivalent amount of DNA (5 µg of DNA: 1 mg of Gold). After this, 100 µl $CaCl_2$ (1 M) was added drop wise to the previous mix while vortexing. Both spermidine and $CaCl_2$ were used to facilitate DNA precipitation and to obtain an even DNA dispersion on the particles.

The mix was allowed to stand for about 10 minutes at room temperature to enable DNA precipitation, after which it was centrifuged. The pellet (DNA microcarriers) were then washed for three times in absolute ethanol, vortexed and centrifuged at 13000×g for two minutes. The supernatant was discarded after every centrifugation. After the final wash, the pellet was resuspended again in absolute ethanol and 8 µl of 0.1 mg/mL polyvinylpyrrolidone (PVP) solution was added to the mix to serve as an adhesive. This suspension was

transferred to a 5 ml polypropylene tube containing 2 ml of absolute ethanol and was well vortexed before being aspired to a polypropilene tube (previously dried on a N_2 station). The suspension was left to settle for 15-20 min at RT. Once the microcarriers were attached to the tube, the supernatant was carefully aspired by pulling the syringe. The tube was rotated for 30-40 seconds to ensure an even distribution of the microcarriers through the tube. The tube containing the microcarriers was led to dry for about 30 min on a N_2 gas station after which it was cut into cartridges of 1 cm length. The cartridges were separated and kept in a plastic flask with a desiccant at 4ºC. Each plastic flask was opened only once to enable the same transfection efficiency for all the experiments.

Genegun transfection

The Helios gene gun (BioRad) contains a cartridge holder and a diffuser that enables the proper dispersion of the bullets and reduces the air pressure directly incident on the slice. The diffuser was first sterilized in absolute ethanol during 5min and then under UV Light until it was completely dried. The genegun was properly mounted with the cartridge holder filled with the cartridges and posteriorly attached to the helium cylinder. For the bombardment, the genegun was placed over each well in a distance of 1.5 cm from the insert and the gas pressure was set for 180 psi. One to two shootings were done per insert to get a proper amount of transfected cells. The biolistic transfection was performed at DIV2, DIV7 and DIV15, depending on the day of the live imaging. Adapted from (Woods and Zito 2008).

STIMULI

Hippocampal organotypic slices (5-6, 14-15, 20-21 DIV) and primary cultures of rat hippocampal neurons (DIV18) were incubated with the GHS-R1a agonist MK-0677 (1 μM) for different time periods. For the immunocytochemistry experiments, MK-0677 was added directly into the conditioned culture medium. For the imaging and biochemistry experiments the conditioned culture medium was totally replaced with fresh culture medium containing MK-0677 (1 μM).

IMMUNOCYTOCHEMISTRY

S3-phosphorilated cofilin (P-cofilin) expression was assessed through imunocytochemistry. For detecting P-Cofilin total expression, imunocytochemistry was performed in fixed cells. Neurons were fixed for 15 min in 4% sucrose and 4% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 and 10 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.4) at room temperature, and permeabilized with PBS + 0.25% (v/v) Triton X-100 for 5 min, at 4ºC. To block non-specific staining, neurons were incubated in 10% (w/v) BSA in PBS for 30 min at 37ºC. The primary antibody diluted in 3% (w/v) BSA in PBS (2 h, 37ºC or overnight, 4ºC) was then incubated. After washing 6 times with PBS, cells were incubated with the secondary antibody diluted in 3% (w/v) BSA in PBS (45 min, 37ºC). Again, after the incubation with the secondary antibodies, the coverslips were carefully washed with PBS. The coverslips were mounted using fluorescent mounting medium from DAKO. Coverslips were sealed on the day after with nail polish. The following primary antibodies were used: anti-P-cofilin, anti-MAP2 and anti-vGlut1. The following secondary antibodies were used: Alexa 488-conjugated anti-rabbit, Alexa 647-conjugated anti-guinea pig, AMCA-conjugated anti-chicken.

IMAGING

HIPPOCAMPAL BANKER NEURONAL CULTURE

Immunostained neurons from primary cultures were imaged using the widefield Axio Observer (Carl Zeiss, Gemany), using a 63× Plan-ApoChromat oil objective (N.A. 1.4). The exposure time was defined to avoid saturation of pixel. A total of 9-13 neurons were imaged per condition.

HIPPOCAMPAL ORGANOTYPICAL SLICE CULTURES

For the dendritic spine studies, hippocampal neurons from organotypical slices were imaged as multiplane (z-series) image stacks on a LSM710 confocal microscope (Carl Zeiss, Gemany) using a 63× Plan-ApoChromat oil objective (N.A. 1.4), zoom 2. For the image acquisition, live neurons were kept in artificial cerebrospinal fluid (ACSF; 127 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM Glucose, supplemented with 4 mM

$CaCl_2$ and 4 mM $MgCl_2$, and 1 uM TTX at the time of the imaging (adapted from (Oliveira and Yasuda 2014)). Secondary dendrites of the apical dendrite of CA1 pyramidal neurons were chosen to be imaged. For the study of spine morphology in the single time point experiments, the intensity of the laser and the gain master were kept similar among acquisitions within experiments. For the time-lapse experiments, the same settings, namely the intensity of the laser and the gain master, were kept equal within each experiment.

IMAGE PROCESSING AND QUANTIFICATION

HIPPOCAMPAL BANKER NEURONAL CULTURE

Images were quantified using image analysis software Fiji. To quantify the total P-cofilin signal, digital images were subjected to a user defined intensity threshold to select clusters and measured for cluster intensity, number, and area for the selected region. The synaptic P-cofilin clusters were selected by their overlap with thresholded Vglut1 signal (synaptic marker).

HIPPOCAMPAL ORGANOTYPICAL SLICE CULTURES

For optimal resolution, the z-stacks images obtained for the studies of spine morphology were deconvolved by Huygens deconvolution software (Scientific Volume Imaging, Hilversum, The Netherlands) using a theoretical point spread function based on microscope parameters. Images were recorded at values according to the Nyquist rate to prevent aliasing. The classic maximum likelihood estimation algorithm was used to deconvolve the confocal images. This algorithm takes the following factors into consideration: NA of the microscope objective; refractive index of the medium; excitation wavelength; emission wavelength; confocal pinhole radius; pixel size; z-axis interval and microscope type.

The number of iterations of the algorithm was set to 70 and the signal to noise ratio of the image was manually defined according to each image.

Spine morphology and head-spine diameter quantification

To classify the different morphologies and the diameter of the spine-head of dendritic spines, NeuronStudio software (Rodriguez, Ehlenberger et al. 2008) was used and the dendritic spines were manually classified accordingly with the following criteria: protrusions without head were identified as filopodia; bulbous spines with no neck were identified as stubby spines; big headed spines with a defined neck were identified as mushroom, cup-shaped spines were defined as branched and spines with identifiable necks and small heads were identified as thin spines.

Spine volume quantification

In this study, we assumed that the spine volume and the amount of fusion protein were proportional to the integrated intensity of the *mCherry* signal (Svoboda 2004). FiJi was used to measure signal intensity on the time-lapse experiments. In these experiments, the number of planes in the z-stack varied among timepoints. Before quantifying the images, we did a sub-stack in order to keep constant the number of planes for each z-stack along the time-lapse experiment. For the quantification, a maximum intensity projection was performed to the z-stack, we thresholded the image and defined the region of interest (ROI) surrounding the dendritic spine head. Over the several time-points of the experiment, the signal from each ROI was followed and measured. To normalize the dendritic spine head signal intensity, a ROI in the secondary dendritic body was done over the successive time-points. A total of 2-7 neurons were imaged per condition in each independent experiment.

BIOCHEMISTRY

PROTEIN EXTRACTS

Hippocampal slices were lysed in radioimmunoprecipitation assay buffer (RIPA; 150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS - sodium dodecyl sulfate and 50 mM Tris, pH 8.0, supplemented with 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), CLAP (1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml pepstatin) as protease inhibitor and 50 mM sodium fluoride (NaF), 1 mM Sodium Ortovanadate (Na_3VO_4) and 1 mM Okadaic Acid ($C_{44}H_{68}O_{13}$) as phosphatase inhibitors. After

mechanic dissociation of slices, they were led to settle for 30 min, at 4ºC, before sonication on ice for 1 min, in cycles of 5 seconds each. After a complete cell disruption, the samples were centrifuged for 5 min, at 4ºC, at maximum speed. The protein extracts in the supernatant were quantified using the bicinchoninic acid (BCA) assay kit (Pierce, Termo Fisher Scientific, Rockford, USA), and the samples were denatured with 5x concentrated loading buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) Glicerol, 2% (v/v) SDS, 0.01% (w/v) NZYTECH bromophenol blue], and boiled at 95ºC for 5 min.

ELECTROPHORESIS GELS AND WESTERN BLOT

Protein extracts were resolved by SDS-PAGE in 12,5% polyacrylamide gels. For western blot analysis, proteins were transferred onto a PVDF membrane (Millipore, Madrid, Spain) by electroblotting (300 mA for 1h30 at 4ºC). The membranes were briefly washed in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% (v/v) Tween-20 (TBS-T) and then blocked for 1 h at room temperature in TBS-T and 5% (w/v) BSA. Membranes were probed overnight, at 4ºC, with the primary antibodies diluted in 5% (w/v) BSA in TBS-T. Following 5 washes of 5 minutes each, 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 Chemidoc (BioRad) and quantified using the ImageJ software under linear exposure conditions. Phosphorylated-cofilin was firstly probed after which the membranes were stripped (0.2 M NaOH for 5 min) and re-probed to Total-cofilin.

STATISTICAL ANALYSES

Statistical differences were calculated according to nonparametric tests for most part of the cases: all graphs represent average values ± S.E.M. Statistical differences were calculated with Prism (Graphpad). Mann-Whitney test, Unpaired t test or Paired t test were used to compare statistical differences between any two groups. Spearman correlation coefficients were carried out to assess the relationship between the initial spine volume vs fold change of spine volume.

Chapter 3
RESULTS

DENDRITIC SPINES: DYNAMICS STRUCTURES IN CA1 HIPPOCAMPAL NEURONS THROUGH DEVELOPMENT

Dendritic spines are thought to be dynamic structures involved in regulating synaptic strength; therefore, it is not surprising that their study both in physiological and pathological situations raises a lot of interest. A small number of studies have shown how dendritic spines evolve during development both *in vivo* and *in vitro* (mainly in primary cultures), but a thorough characterization of dendritic spine dynamics throughout development is lacking in the literature. Hippocampal organotypic slices preserve the synaptic organization of the hippocampus, critical to the understanding synapse function in a more naturalistic context, and have been increasingly used as a model for synaptic studies, such as the one of this thesis work. Therefore, as a starting point for this work, we sought to characterize dendritic spine dynamics in hippocampal slices over development. The parameters used to characterize dendritic spines were the following: morphology, number and head diameter.

For the morphological experiments, protrusions without head were identified as filopodia; bulbous spines with no neck were identified as stubby spines; cup-shaped spines termed as branched; big headed spines with a defined neck were identified as mushroom and spines with identifiable long necks and small heads were identified as thin spines (Figure 8A). To characterize the different stages of spine development, we performed these analyses in hippocampal slices at three different developmental stages: DIV5-6, DIV14-15 and DIV20-21. For the earlier stage of development, we biollistically transfected hippocampal slices at DIV2 with a plasmid encoding the red fluorescent protein *mCherry*, and imaged the transfected neurons with a confocal microscope at DIV5-6. For older stages of development, the transfection was performed 7 days before the imaging, which was done at DIV14-15 or DIV20-21 cultures (Figure 8B and 8C). For these analyses, we chose to live-image spines from secondary apical dendrites of CA1 pyramidal neurons, as they have been the subject of many studies on synaptic and cognitive function, and mainly because previous studies in our lab have observed effects of the GHS-R1a activation in this hippocampal region, which is the main focus of this thesis.

Our morphological analyses showed that the secondary dendrites of CA1 neurons of DIV5-6 slices presented a dominance of the population of mushroom spines (40.04±8.46%), followed by stubby spines (27.05±12.6%), thin spines (23.92±11.38%) and filopodia (8.47±6.73%(SD)) (Figure 8D). In slices ten days older (DIV14-15), the proportion of thin

(27.59±8.792%) and stubby (22.37±8.611%) spines was similar, while mushroom spines represented a bigger fraction of the spine population (45.17±11.06%). Filopodia (4.426±8.37%) and branched spines (3.618±6.129%) represented a smaller fraction (Figure 8D). Older slices (from DIV20-21 slices) present a large proportion of mushroom spines (61.59±11.53%) followed by thin (22.18±9.56%) and stubby (14.41±8.12%) spines and filopodia (1.81±2.60%) (Figure 8D). Regarding the overall spine density and spine head diameter, our data showed that the dendritic spine density was similar among the different developmental stages studied, being approximately 4 spines per 10 μm of dendritic length. Similarly, the head diameter of spines remained around 0.7 μm in all the different developmental stages.

Taken together, our morphologic analyses of spines showed that CA1 neurons are mainly composed of mature spines (thin, mushroom and stubby) for all the considered developmental stages. It is also interesting to observe that the percentage of filopodia structures, the precursors of spines, decreased over the different developmental stages being 8.47±6.73% in DIV5-6 slices, in comparison with DIV14-15 (3.98±3.37%) and with DIV20-21 (1.81±2.60%). When taking into consideration the overall spine density and the overall head diameter of spines, our results suggest that spines can be considered as stable structures over development. Nevertheless, the reduction in filopodia, the precursors of spines, and an increase in the mushroom spines, suggest that CA1 neurons, in hippocampal slices, become less plastic over development.

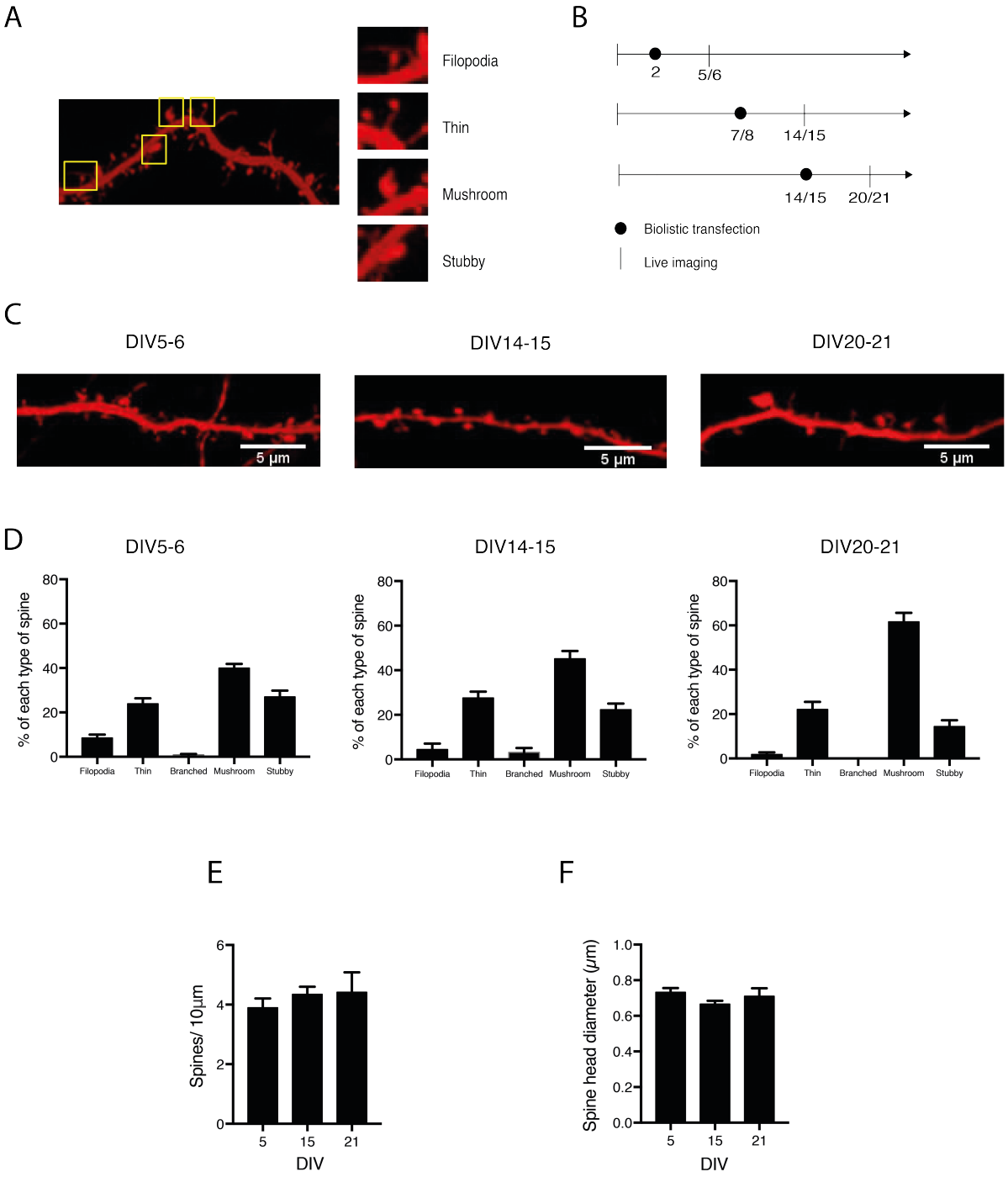


Figure 8. Dendritic spines were dynamic structures in CA1 hippocampal neurons through development. A. Representative images of filopodia and spines in a dendritic segment. The yellow squares identify spines with different morphologies (left), which can be seen at a higher magnification in the right. The protrusions were classified by size and shape as filopodia (percursors of spines), mushroom, thin, branched and stubby spines. Filopodia are long protrusions without an identifiable head. Mushroom spines have a bulbous head diameter and a narrow neck. Thin spines have a small head and a long neck, and their overall length exceeds their width. Branched spines posses a cup-shape. Stubby spines do not have a neck. **B.** Timeline for the experiments. Organotypic hippocampal slices were biollistically transfected with a plasmid encoding *mCherry* (a neuronal filling marker) at DIV2, DIV7-8 and DIV14-15. CA1 pyramidal neurons were live imaged in ACSF at the confocal microscope at DIV5-6, DIV14-15 and DIV20-21, respectively. **C.** Representative images of spines from secondary apical dendrites of CA1 pyramidal neurons at different DIVs. Scale bar, 5 *µ*m. **D.** The density of each spine morphology changes during development. In DIV5-6 hippocampal slices,

23.92±11.38% of the spines were thin and 27.05±12.60% of the spines were stubby. Mushroom spines represented 40.04±8.46% of the spines. In DIV14-15 slices, 27.59±8.79% of the spines were thin, 22.37±8.61% were stubby, and 45.17±11.06% were mushroom. In DIV20-21 slices, 22.18±9.59% of the spines were thin, 14.42±8.12% of the spines were stubby and 61.59±11.53% of the spines were mushroom. Filopodia-like structures were more abundant at DIV5-6 (8.469±6.732%) in comparison with DIV14-15 (4.426±8.37%) and with DIV20-21 (1.811±2.603%). Results are expressed as number of each type of spine per total number of spines. **E.** Spine density remained similar (4 spines in 10μm) at the different developmental stages studied. Results are expressed as number of spines per dendritic length. **F.** The head diameter of spines was around 0,7μm. Results are expressed as an average of the head diameter of spines. **B-F.** Data are from 10 neurons from 4 independent experiments for the DIV20-21 experiments; 24 neurons from 4 independent experiments for the DIV14-15 (in E and F; but only 10neurons from 2 independent experiments in D) and 21 neurons from 4 independent experiments for the DIV5-6 experiments. Quantitative analyses were performed with NeuronStudio software. Error bars represent S.E.M.

LONG-TERM ACTIVATION OF THE GHS-R1a DOES NOT AFFECT OVERALL SPINE MORPHOLOGY OR DENSITY IN HIPPOCAMPAL SLICES

Previous work from our lab showed that the long-term GHS-R1a activation with its agonist (MK-0677 1µM, for 20h) induced an increase in the number of clusters of PSD-95 co-localized with Vglut1 (post- and pre-synaptic markers, respectively) and of F-actin, in cultured hippocampal neurons as assessed by immunocytochemistry (Joni van Leeuwen Master's thesis, 2013). Furthermore, long-term GHS-R1a activation with its agonist (MK-0677 1µM, for 20h) induces activity-dependent GluA1-AMPAR synaptic delivery in CA1 hippocampal neurons and enhances NMDAR-dependent LTP in the hippocampus, as assessed by electrophysiology studies in organotypic slices (Ribeiro, Catarino et al. 2014). Taken together, these results seem to suggest that a prolonged activation of the GHS-R1a play a role in synaptic plasticity and possibly in spine dynamics as well, which led us to wonder about the role of the long-term GHS-R1a activation in our model of study. In addition to the previously described characterization of the spine morphology during development, we sought to investigate how the GHS-R1a activation could affect the relative proportion of spine's morphology populations at each developmental stage. To test this, we biollistically transfected organotypic hippocampal slices with the plasmid encoding *mCherry* (a neuronal filling marker) at DIV2, DIV7-8 and DIV14-15. We decided to use the GHS-R1a agonist, MK-0677, to mimic the effects of ghrelin in spine dynamics, for two reasons: 1) ghrelin half-life is about 30min, a very short period for some of our experiments requiring long periods of incubation; 2) previous experiments in our lab had used the GHS-R1a agonist. The slices were incubated with the GHS-R1a agonist MK-0677 (1 μM) for ~20 h before neurons were live imaged at DIV5-6, DIV14-15 and DIV20-21, respectively (Figure 9A). Our results showed that the GHS-R1a activation with the agonist did not affect the density of any type of mature spines (thin, mushroom and stubby) in our cultures over the different

stages of development (Figure 9B). Similarly, the ghrelin receptor activation did not affect the overall density (Figure 9C) nor the average head diameter (Figure 9D) of the spines.

Taken together, these results showed us for the first time that the long-term GHS-R1a activation did not affect overall dendritic spine morphology or density in hippocampal slices in any of the development stages studied.

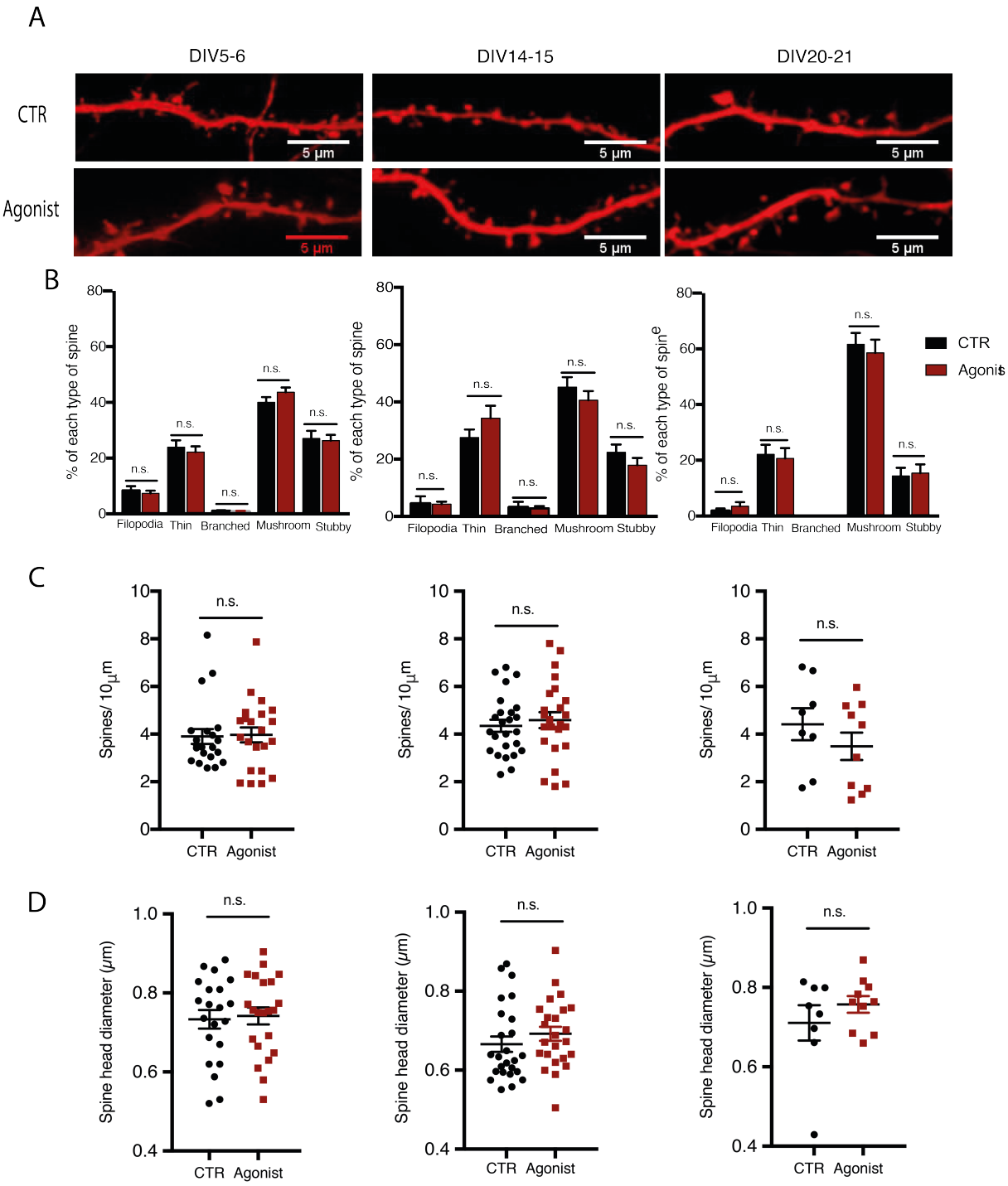


Figure 9. Long-term activation of the GHS-R1a did not affect overall spine morphology or density in hippocampal slices. A. Representative images of spines from secondary apical dendrites of CA1 pyramidal neurons at different DIVs both from control (CTR) and agonist conditions (Agonist). Hippocampal slices were biollistically transfected with a plasmid encoding *mCherry* (a neuronal filling marker) at DIV2, DIV7-8 and DIV14-15. The slices were incubated with the GHS-R1a agonist MK-0677 (1 μM) for ~20 h before neurons were live imaged at DIV5-6, DIV14-15 and DIV20-21, respectively. Scale bar, 5 μm. **B.** Effects of long-term activation of the GHS-R1a on spine morphology for different ages. The density of each type of spine did not change between control and agonist-incubated slices fom cultures of all ages. **C.** Incubation with the GHS-R1a agonist for 20h did not alter the overall spine density. Results are expressed as number of spines per dendritic length. **D.** Incubation with the GHS-R1a agonist for 20h did not alter the total spine head diameter. Results are expressed as an average of the head diameter of spines. **B-D.** Data are from 10 neurons per condition from 4 independent experiments for the DIV20-21 experiments; 24 neurons per condition from 4 independent experiments for the DIV14- 15 for the analyses of the total spine density and the total head diameter (C and D); 10neurons per condition from 2 independent experiments for the DIV14-15 (B). Quantitative analyses were performed with NeuronStudio software. Results are presented as means ± S.E.M. Statistical significance was determined by the Mann–Whitney test (n.s. P >0.05).

SHORT-TERM GHS-R1a ACTIVATION AFFECTS SPINE DYNAMICS IN HIPPOCAMPAL SLICES

Ribeiro et al. (2014) observed that a short-term GHS-R1a activation for 1h with the GHS-R1a agonist (MK-0677, 1 μM) in cultured hippocampal neurons increased the cell surface expression of GluA1-AMPA receptors, as assessed by imunocytochemistry. Moreover, van Leeuwen (2013) showed that the same stimulus not only increased the intensity of vGlut1 and PSD95 clusters but also increased the total area of F-actin clusters in mature hippocampal neurons. Taking all of this into consideration, we hypothesized that the activation of the GHS-R1a with its agonist (MK-0677, 1 μM) for a short period of time (50 min) could trigger changes on dendritic spines. To better explore this hypothesis, we decided to follow each spine individually in basal conditions or after stimulation with the GHS-R1a agonist, and to study the spine head volume changes over time, instead of characterizing overall spine morphology, as in the previous experiments.

As we did not know the behaviour of spines over a short time period, we felt the need to start by the characterization of spine dynamics in basal conditions in hippocampal slices over a period of 80 min. Figure 10A depicts a scheme of this experiment. Hippocampal slices were biollistically transfected at DIV2 with the *mCherry* plasmid and neurons were imaged with a confocal microscope at DIV5-6. Secondary apical dendrites of CA1 neurons from hippocampal slices were imaged every 10min during a 80 min period (Figure 10B). Our results showed that the average spine head volume did not suffer changes over the 80 min of imaging (Figure 10C). Nevertheless, spines behave dynamically, as 52.74±28.36% of the

spines shrunk (the change of spine volume relative to the baseline – determined as the average spine volume for the first three time points - is inferior to 0.95%), 30.93±10.66% of the spines grew (the change of spine volume is superior to 1.05%) and 16.33±12.23% of the spines did not shrink or grow (the fold change of spine volume relative to the baseline ranges from 0.95-1.05%) (Figure 10D).

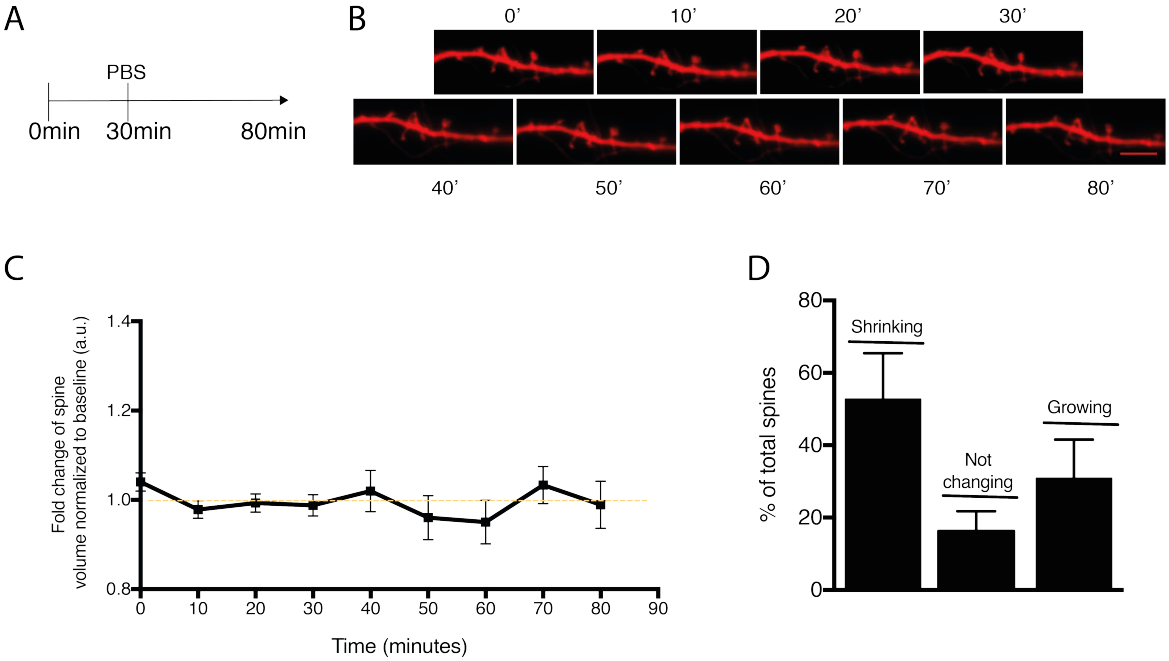


Figure 10. Spines were dynamic during a 80 min imaging period in hippocampal slices (DIV5-6). A. Timeline of the experiment. CA1 neurons from hippocamapal slices (DIV5-6) were imaged every 10 min during a 80 min time lapse experiment. **B.** Representative images of a single dendritic section over 80 min. Scale bar, 5 μm. **C.** The average spine head volume does not change over 80 min of the time-lapse experiment. **D.** After 80 min, 52.74±28.36% of the spines shrunked compared to their initial volume (spine volume change is inferior to 0.95% in comparison to the baseline); 30.93±10.66% grew (spine volume change is superior to 1.05%) and 16.33±12.23% of the spines did not shrink nor grow (spine volume change is between 0.95-1.05% of the baseline volume). **C.** Data are from 90 spines from from DIV5-6 hippocampal slices from 5 independent experiments. **D.** Data is from 5 neurons from 5 independent experiments. Quantitative analyses were performed with FiJi software. Results are presented as means ± S.E.M.

To study the short-term effects of the GHS-R1a activation on hippocampal slices, we imaged each dendrite in ACSF for 30 min before stimulus (basal) and for 50 min after stimulus with either the agonist of the receptor (MK-0677 1μM) or vehicle (PBS) (Figure 11A,B). Our results showed that GHS-R1a activation with the agonist (MK-0677, 1 μM) significantly increased the spine head volume after 50 min of incubation when comparing to the initial spine volume for each spine (-30 min,*p<0,05, Paired t' test), but not after vehicle incubation (n.s, Paired t' test) (Figure 11C). Interestingly, after 50 min incubation with the GHS-R1a agonist, we observed a decrease in the number of spines that shrunk (CTR: 52.74±28.36%; MK-0677: 42.69±21.27%), an increase in the number of spines that grew (CTR:

30.93±10.66% ; MK-0677: 49.26±16.94%), and a decrease in the number of spine that did not shrink or grow (CTR: 16.33±,12.23%; MK-0677: 8.0±5.9%), when comparing to the control (Figure 11D).

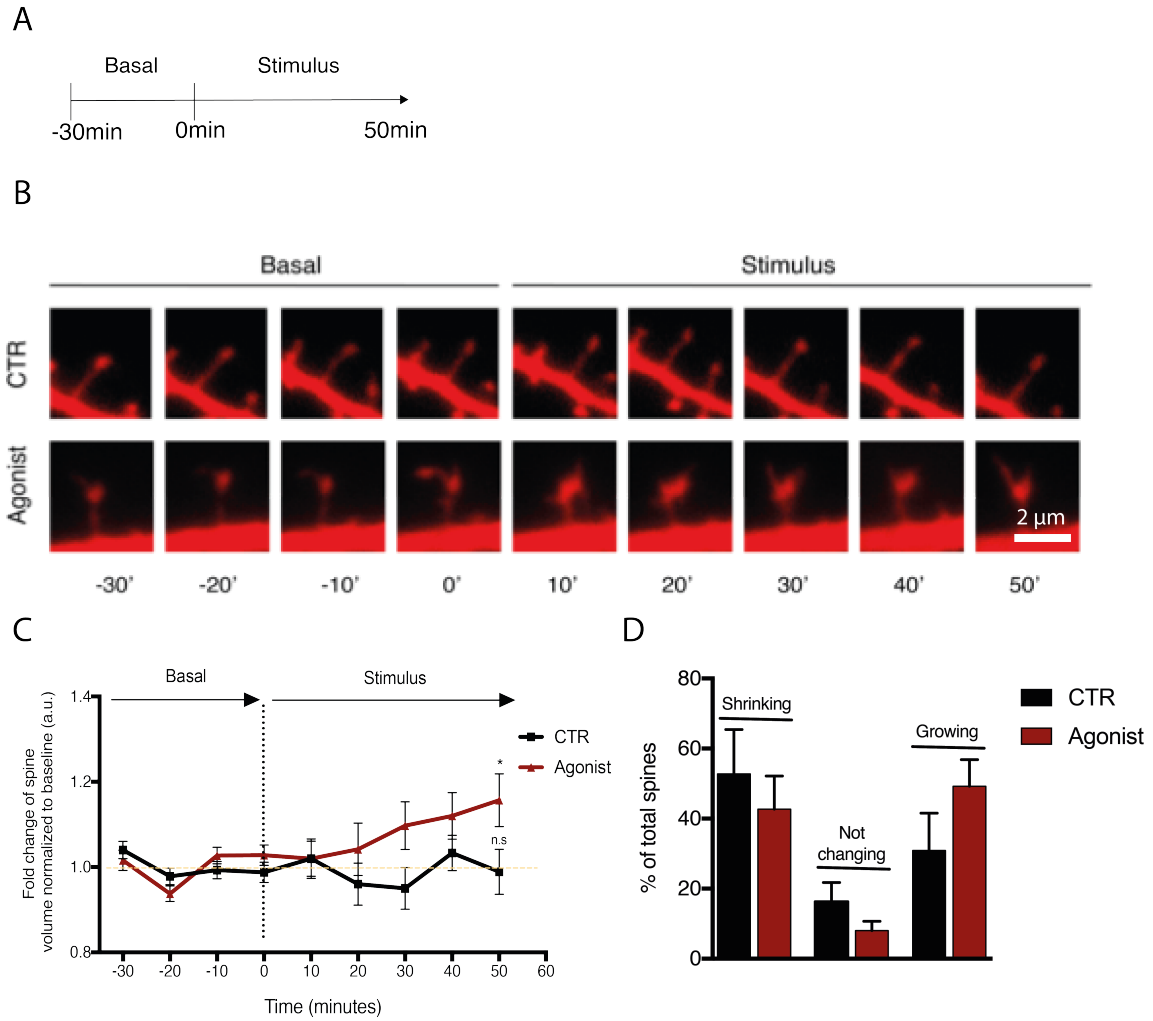


Figure 11. Short-term GHS-R1a activation affected spine dynamics in hippocampal slices (DIV5-6). A. Dendritic spines were sampled for 80 min at 10 min intervals from control (CTR) and Agonist (1μM MK-0677)-treated hippocampal slices. Each dendrite was imaged in ACSF for 30 min before the stimulus (basal) and for 50min after stimulus with either MK-0677 (1 μM) or vehicle (PBS). **B.** Representative images of a single spine over the period of the experiment. Scale bar represents 2μm. **C.** GHS-R1a activation increased the overall head volume of spines after 50 min of incubation with the agonist, when comparing to the control (*p<0,05, unpaired t test). **D.** GHS-R1a activation altered spine dynamics as the number of spines that shrunk and that remained stable was reduced while the number of spines that grew increased, in comparison with the control. **C.** The head volume of each spine was quantified with FiJi software and normalized to the baseline spine volume (averaged for each spine from its head volume for the first three time points of the experiment). Results are expressed as fold change of baseline. Data for the control condition are from 90 spines for all timepoints from DIV5-6 hippocampal slices from 5 independent experiments. Data for the agonist condition are from 147 spines in the 0-10 min and 40-80 min timepoints, 130 spines for the 20 min timepoint and 118 spines for the 30 min timepoint from DIV5-6 hippocampal slices from 5 independent experiments. **D**. Data is from 5 neurons for each condition, from 5 independent experiments. Quantitative analyses were performed with FiJi software. All the results are presented as means ± S.E.M. Statistical analysis was performed using the a paired t test (*p<0.05; n.s. p>0.05). Where not mentioned, significant statistical differences were not found.

These results showed that activation of the GHS-R1a increases the spine head volume and suggest that this effect is partially due to a shift in both the population of spines that grow (there are more spines growing upon incubation with the agonist) and the population of spines that shrink (there are less spines shrinking upon incubation with the agonist). It is interesting to observe that the incubation with the GHS-R1a agonist also decreased the population of spines that did not shrink or grow, and if we consider that this is the population of stable spines, our results suggest that the activation of the GHS-R1a decreases the stability of spines. Overall, these results suggest that the GHS-R1a activation may affect structural plasticity by interfering with the three types of spine populations.

SMALL SPINES ARE MORE RESPONSIVE TO THE GHS-R1a ACTIVATION THAN LARGE SPINES IN HIPPOCAMPAL CA1 NEURONS

The GHS-R1a activation affected the number of spines that shrink, that grow or that do not shrink or grow. However, is there a population of spines more prone to change than others? For example, in the case of synaptic potentiation, protein synthesis independent plasticity occurs preferentially at smaller spines (Matsuzaki, Honkura et al. 2004), and leads to short term structural modifications (1.5 h), while long lasting, protein synthesis dependent stimuli lead to correspondingly longer lasting structural changes (4 h) on spines of various sizes (Matzusaki et al, 2004). To better understand what is happening in our system, we used a heat map to analyse the data and observe the magnitude of the differences in spine head volume for each spine separately (Figure 12A and 12B). The visual organization of the data in this map enabled us to observe that small spines were more responsive to the GHS-R1a activation and that this effect was more expressive with longer incubation times (Figure 12A and 12B).

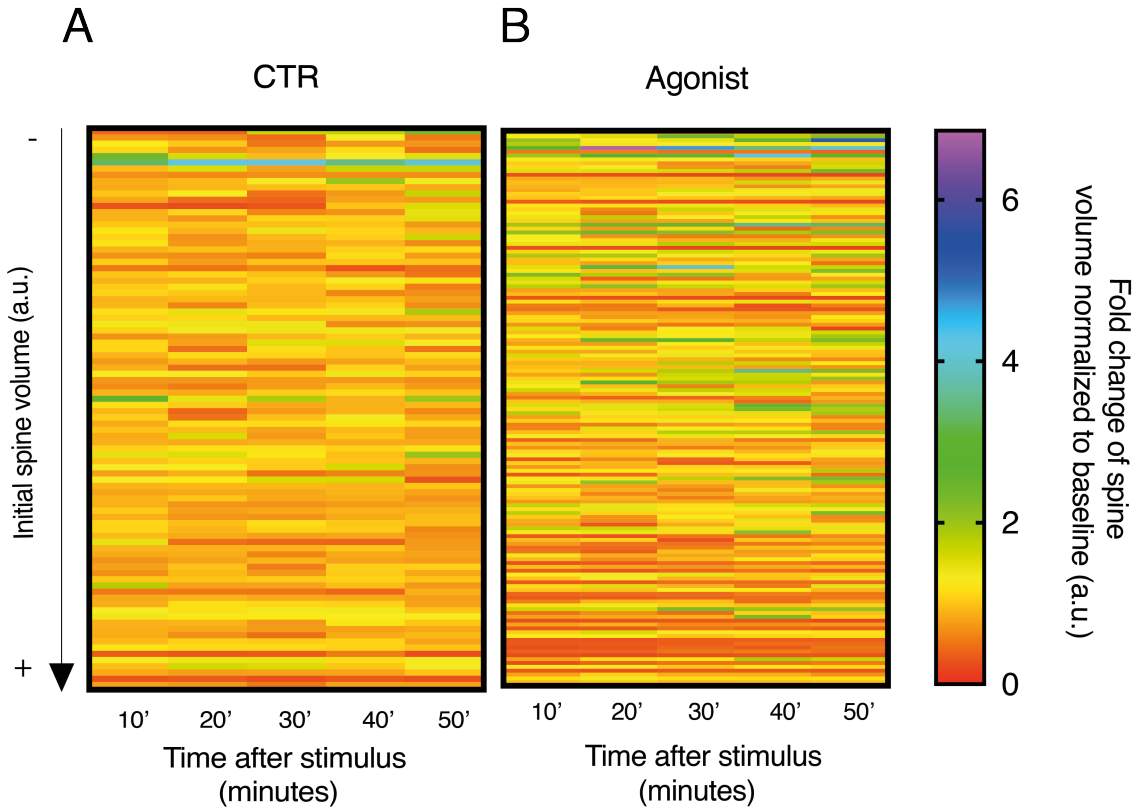


Figure 12. GHS-R1a activation increased the spine head volume mainly of small spines. Heat maps were generated with a data set of the fold change of spine volume normalized to baseline, after incubation with the stimulus for all analysed spines (PBS-control or with MK-0677 1μM). The initial volumes of the spine heads were sorted from the smallest (top of the map) to the largest (bottom of the map). **A.** The heat map on the left depicts the fold changes of the spine head volumes in the control condition; **B.** The heat map on the right depicts the fold changes on the spine head volumes in the condition incubated with the agonist. Color bars reflect the fold changes in dendritic spine volumes. **A-B.** Data for the control condition are from 90 spines for all timepoints from DIV5-6 hippocampal slices from 5 independent experiments. Data for the agonist condition are from 147 spines in the 0-10 min and 40-80 min timepoints, 130 spines for the 20 min timepoint and 118 spines for the 30 min timepoint from DIV5-6 hippocampal slices from 5 independent experiments.

Since the increase in the spine head volume was more evident for longer incubation times, we decided to focus on the last time-point imaged to check whether the initial spine head volume could correlate with the volume fold change observed after 50 min of incubation with the agonist. Figure 13A depicts the plot for the initial spine head volume vs the volume fold change and shows that small spines suffered bigger changes in volume, in comparison to the baseline and to the control. To further understand how spines of different volumes responded to the GHS-R1a activation over the time course of the experiment, we started by defining what we consider to be a "small" or a "large" spine. "Large" spines were defined as the spines above the median spine head volume while the "small" spines were defined as the spines below the median spine head volume. Figure 13A shows that smaller spines were more responsive to GHS-R1a activation in comparison with the control, since there was a stronger negative correlation between the two variables (initial spine volume and fold change of spine volume) for the agonist-treated cultures than for the control (r=-0.2814, for the

agonist, p=0.0006; r=-0.1073, for the control, p=3142, Spearman rank correlation). Small spines from slices incubated with the agonist for 30 min showed a significant increase in spine head volume (*p<0.05, unpaired t test) in comparison to the control. Small spines from agonist-incubated slices significantly increased in volume after 10 min (#p<0.05, paired t test), 30 min (##p<0.01, paired t test), 40 min (#p<0.05, paired t test), and 50 min (#p<0.05, paired t test), compared to the baseline (Figure 13B). Large spines from slices incubated with the agonist for 50 min did not show a significant change in spine head volume, in comparison to the baseline (Figure 13C). On the other hand, large spines from slices incubated with the vehicle for 50 min showed a significant decrease in spine head volume (*p<0.05, paired t test), in comparison to the baseline (Figure 13C). However, in both populations of spines, there was a decrease in the number of the spines that shrunk, after GHS-R1a incubation with the agonist for 50 min, when comparing to control (Figures 13D, E). Also, for both populations, there was an increase in the number spines that grew (Figures 13D, E) but this effect was bigger when considering large spines. Furthermore, the pool of spines that did not shrink or grow remained unchanged in the small spines population and decreased in the large spines population (Figures 13D, E).

Taken together, these results suggest that not all spines responded the same way to the GHS-R1a activation, rather, there is a selective population of spines, in this case, the small spines, that are more responsive to the GHS-R1a activation since they showed larger volume changes upon incubation with the agonist. As observed in Figure 13, the overall spine head volume changes were not only due to effects in the magnitude of the volume of each spine head but also due to the number of spines affected by the stimulus. Moreover, our results showed that the spine head volume of large spines decreased over the time of the experiment, such that, after 80min, there is a statistical significance between conditions (Figure 13C), an effect that is not observed when the slices were incubated with the agonist. These results seem to suggest that the GHS-R1a activation may be involved in the stabilization of large spines.

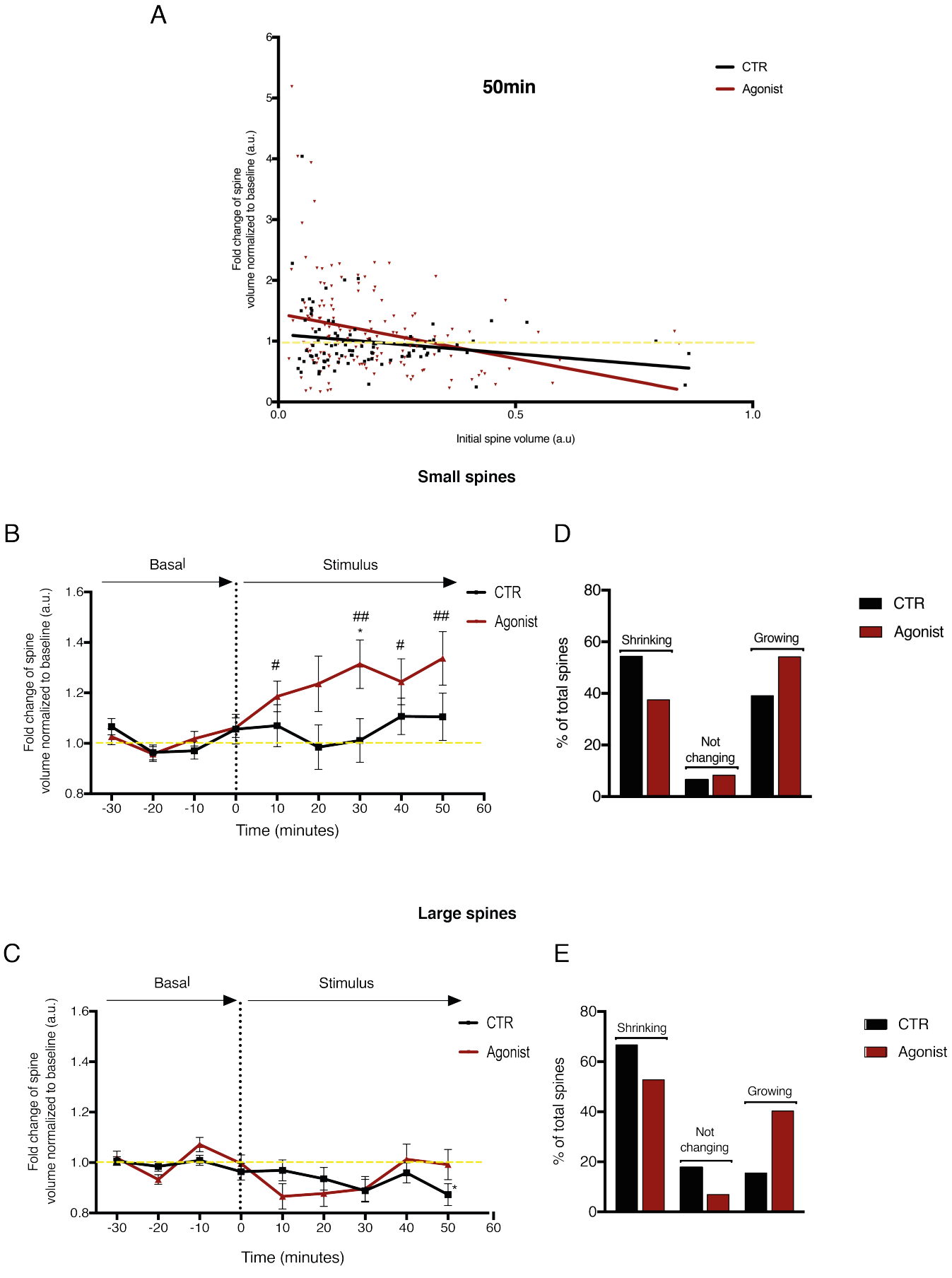
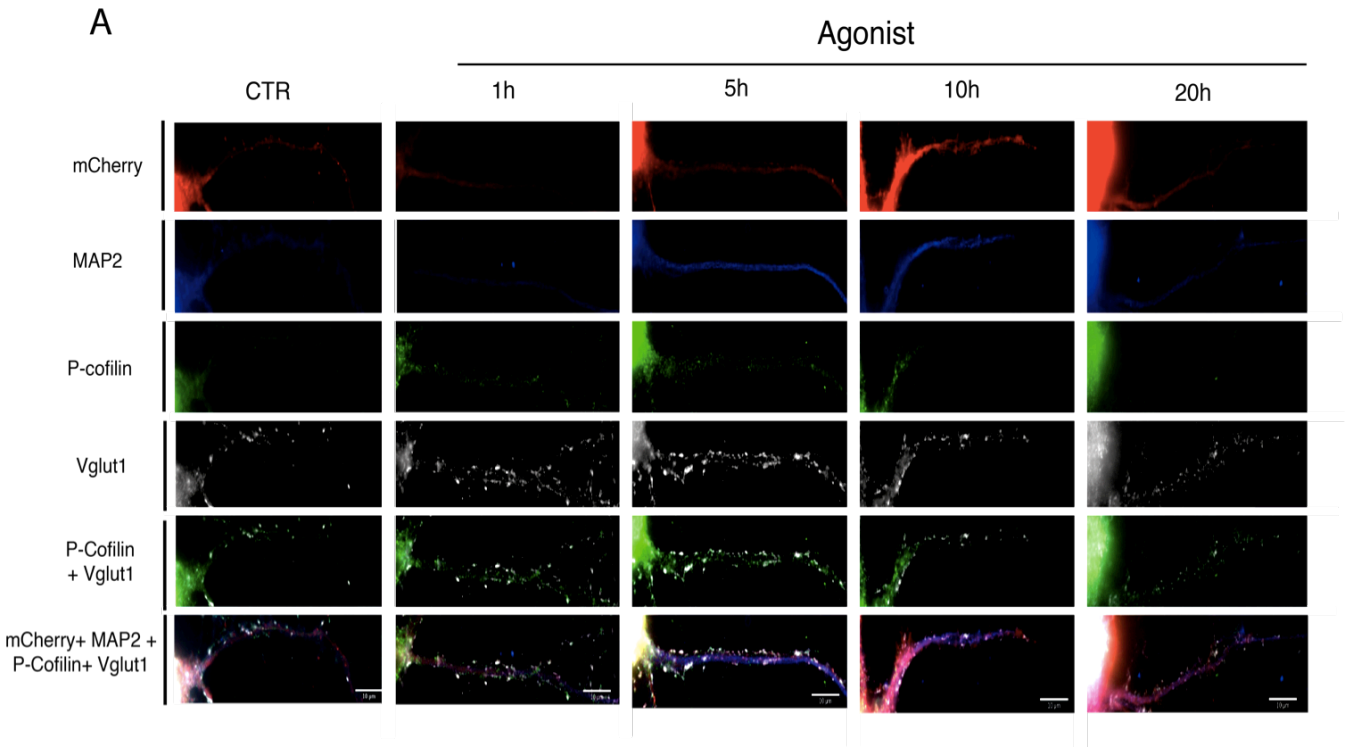

A
50min
CTR
Agonist
Fold change of spine volume normalized to baseline (a.u.)
Initial spine volume (a.u)
Small spines
B
Basal
Stimulus
CTR
Agonist
Fold change of spine volume normalized to baseline (a.u.)
Time (minutes)
D
% of total spines
Shrinking
Not changing
Growing
CTR
Agonist
Large spines
C
Basal
Stimulus
CTR
Agonist
Fold change of spine volume normalized to baseline (a.u.)
Time (minutes)
E
% of total spines
Shrinking
Not changing
Growing
CTR
Agonist

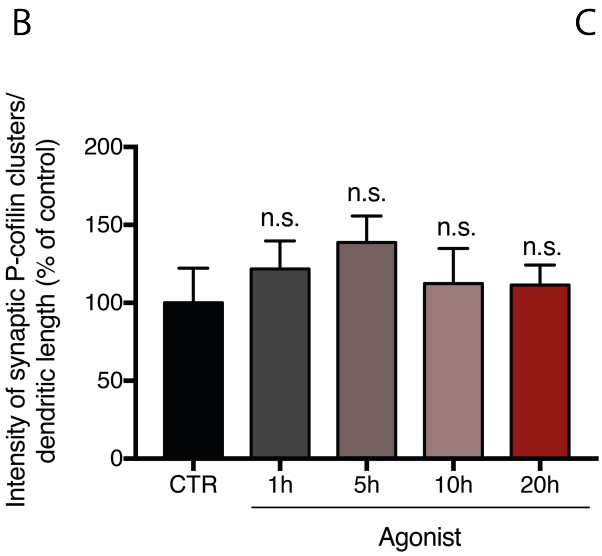
Figure 13. Small spines were more responsive to GHS-R1a activation than large spines in hippocampal CA1 pyramidal neurons. A Linear regression of initial spine volume vs. magnitude of change, at 50 min after the agonist incubation. The initial volume of the spines affects the response of dendritic spines to GHS-R1a activation. It was found a significant negative correlation between the initial spine volume and the magnitude of change in response to activation of the GHS-R1a (r=-0.2814, p=0.0006), whereas in control conditions no correlation was found between changes in spine volume and the initial spine volume (r=-0.1073, p=3.142). **B.** Time course of the experiment plotting only the population of the small spines group (all the spines below the median volume). Spines from CA1 neurons of hippocampal slices incubated with the agonist for 30 min showed a significant increase in spine head volume (*p<0.05, unpaired t test) in comparison to the control. Spines from agonist-incubated slices were significantly increased after 10 min (#p<0.05, paired t test), 30 min (##p<0.01, paired t test), 40 min (#p<0.05, paired t test), and 50 min (#p<0.05, paired t test), comparing to the baseline. **C.** Time course of the experiment plotting only the large spines group (all the spines above the median volume of spine head). The spines from slices incubated with the agonist for 50 min did not show a significant decrease in spine head volume as observed for control spines (*p<0.05, paired t test), in comparison to the baseline. In the graph, the black dotted trace represents the moment when the stimulus was applied. **D, E.** GHS-R1a activation with the agonist (MK-0677, 1 μM) altered the dynamics of large and small spines as the number of spines that shrunk and that remained stable was reduced while the number of spines that grew increased, in comparison with the control. **B-E.** Data for the control condition are from 90 spines for all time-points. Data for the agonist condition are from 147 spines in the 0-1 0min and 40-80 min time-points, 130 spines for the 20 min time-point and 118 spines for the 30 min time-point. Quantitative analyses were performed with FiJi software. Results are presented as means ± S.E.M. Statistical significance was determined by the Unpaired t test or a paired t-test (*p < 0.05, unpaired t test; #p<0.05; ##p<0.01, paired t test; n.s. p>0.05). Where not mentioned, significant statistical differences were not found.

GHS-R1a ACTIVATION INREASES THE LEVELS OF PHOSPHORYLATED COFILIN, IN CULTURED HIPPOCAMPAL NEURONS AND IN HIPPOCAMPAL SLICES

Structural changes that can be observed upon activation of a cell membrane receptor, such as the GHS-R1a, are associated to signalling pathways triggered by the activation of that receptor. So far, this thesis work has focused on the study of the effects of ghrelin on the structural level but the molecular mechanisms involved in these changes remain unclear. To further understand the molecular players that could be mediating these structural effects, we chose to study cofilin, an important modulator of actin polymerization. Different post-translational modifications of cofilin play different roles. A well-studied cofilin modification is phosphorylation on S3A, which is thought to affect the activity of cofilin in severing actin filaments, which in turn is involved in spine remodelling. We therefore asked if cofilin phosphorylation was altered upon GHS-R1a activation. To perform this experiment, we used different times of incubation because previous work from our lab showed that several signalling pathways were activated depending on the time of incubation with the agonist. Hence, this experiment helped us not only to study the molecular mechanisms involved in spine dynamics upon GHS-R1a incubation that we observed previously, but also to elucidate

how the GHS-R1a activation affects spine dynamics when it is activated for long periods. This information can contribute for the understanding of the mechanistic and the physiological role of ghrelin. To explore the effect of GHS-R1a activation on the total and synaptic expression of P-cofilin, hippocampal neurons (DIV11) were transfected with the plasmid encoding *mCherry* to allow the visualization of neuronal morphology and to identifiy separately dendrites from axons. Neurons were treated at DIV18 with the GHS-R1a agonist (MK-0677, 1 μM) for different periods of time. Neurons were fixed at DIV19, because at this age of the culture the synaptic contacts are formed and the culture resembles closely to a mature level of development. After its fixation, neurons were stained for P-cofilin, for MAP2 to visualize the dendritic structure and for Vglut1 to visualize synapses (Figure 14A). P-cofilin puncta were quantified, and we verified that the treatment with the agonist significantly increased the total fluorescence intensity of P-cofilin at 5 h incubation with the GHS-R1a agonist (*P<0.05, Mann-Whitney test) (Figure 14C). The number and the area of P-cofilin clusters were significantly increased upon GHS-R1a activation with the agonist both in synaptic and extra-synaptic pools (VGlut1-colocalized, *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001, Mann-Whitney test) (Figure 14D-G). Overall, these results suggested that the signalling pathways activated by the GHS-R1a affect the total and synaptic levels of phosphorylated cofilin.


A
Agonist
CTR
1h
5h
10h
20h
mCherry
MAP2
P-cofilin
Vglut1
P-Cofilin
+ Vglut1
mCherry+ MAP2 +
P-Cofilin+ Vglut1


B
Intensity of synaptic P-cofilin clusters/
dendritic length (% of control)
200
150
100
50
0
n.s.
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CTR
1h
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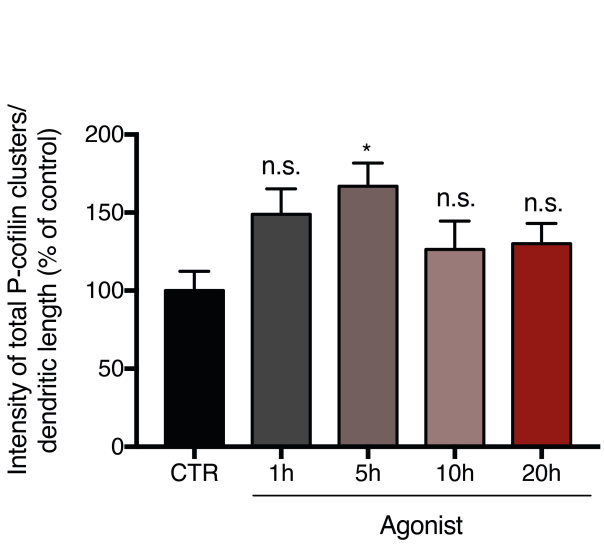
20h
Agonist


C
Intensity of total P-cofilin clusters/
dendritic length (% of control)
200
150
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1h
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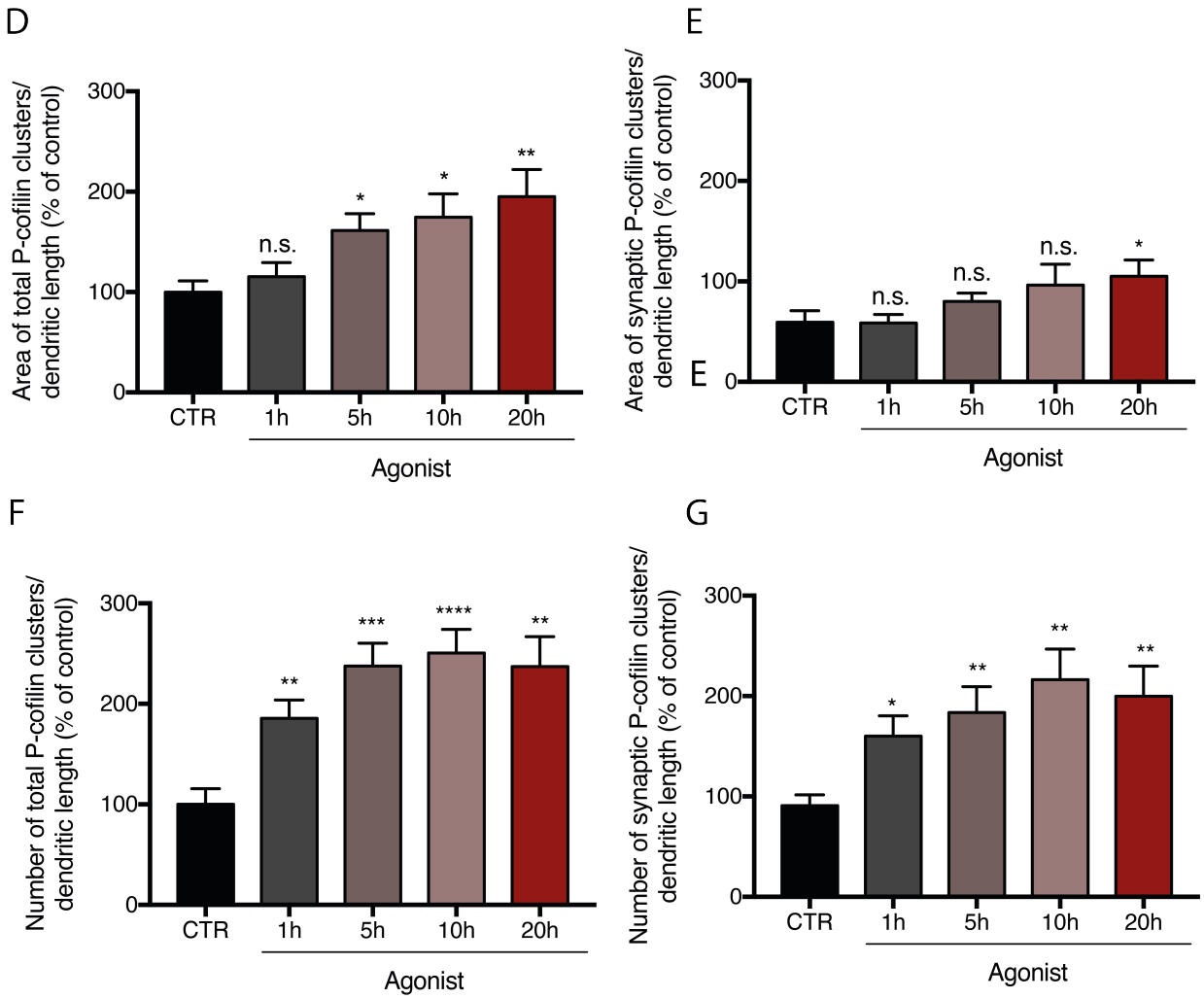

Figure 14. GHS-R1a activation increased the levels of phosphorylated cofilin, in mature hippocampal neurons. A. Hippocampal neurons in culture were transfected at DIV11 with mcherry (red) construct. At DIV18, hippocampal neurons in culture were incubated with MK-0677 (1μM) at different time-points, after which they were fixed and stained for Phosphorylated-cofilin (P-cofilin, green), MAP2 (blue) and VGlut1 (white). Scale bars represent 10 µm. Neurons were analyzed for the total intensity, area and number of total (**B, D** and **F**, respectively) and synaptic (VGlut1-colocalized); **C, E, G,** respectively) cell puncta of P-Cofilin per dendritic length. Quantitative analyses were performed with FiJi software. **B-G.** Results are presented as % of control ± S.E.M. and are averaged from one single experiment (n=9-10 cells; *p<0.05, n.s. p>0.05, Mann-Whitney test).

We next asked if we could see the same pattern of expression of phosphorylated cofilin in the model where we performed the experiments regarding spine dynamics, the organotypic hippocampal slices. We incubated hippocampal slices (DIV20-21, to parallel with the immunocytochemistry experiments) with the GHS-R1a agonist for different time-points and analysed total protein extracts. Our results showed a tendency for an increase in the total expression of P-cofilin and total cofilin after 5 h incubation with the GHS-R1a agonist (Figure 15A-C), similarly to what was observed in hippocampal neuronal cultures.

These results show that the activation of GHS-R1a exerts posttranslational modifications on cofilin, inducing its phosphorylation. Nevertheless, the expression of total cofilin is also (non-significantly) increased. This suggests that the GHS-R1a activation also affects translation of cofilin, or its turnover. It seems that cofilin is phosphorylated mainly after 5h of incubation, which is when the PKC pathway is also activated (Luis Ribeiro Phd Thesis, unpublished work). However, we observed a tendency for an effect of the agonist on phosphorylated cofilin after 1h of incubation, which is when we observed changes in dendritic spines, both in the immunocytochemistry experiments in cultured neurons and in the biochemistry experiments in organotypic hippocampal slices. Overall these data suggest cofilin as a possible player on the structural changes observed in spines upon activation of the GHS-R1a.

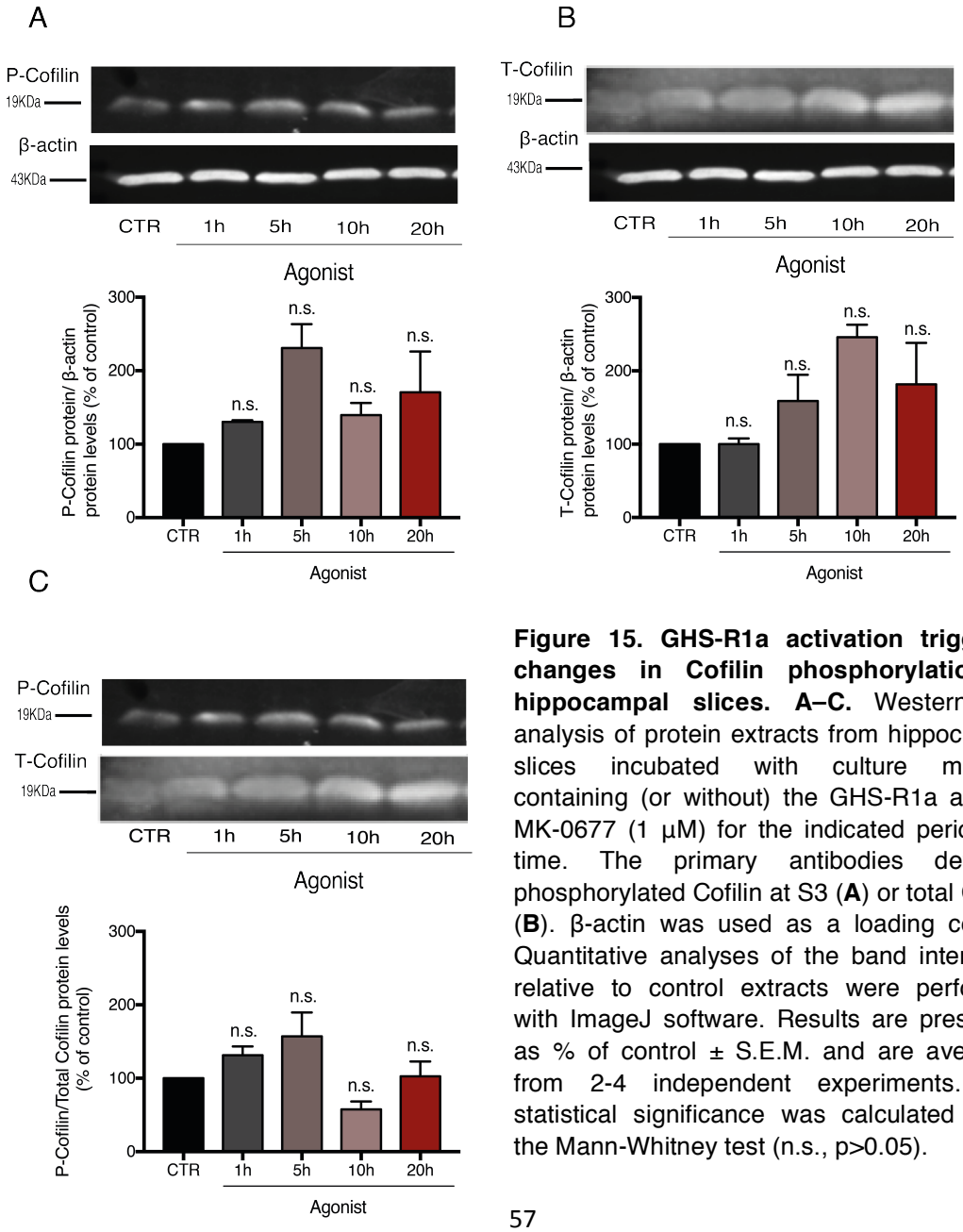

Figure 15. GHS-R1a activation triggered changes in Cofilin phosphorylation in hippocampal slices. A–C. Western blot analysis of protein extracts from hippocampal slices incubated with culture medium containing (or without) the GHS-R1a agonist MK-0677 (1 μM) for the indicated periods of time. The primary antibodies detected phosphorylated Cofilin at S3 (**A**) or total Cofilin (**B**). β-actin was used as a loading control. Quantitative analyses of the band intensities relative to control extracts were performed with ImageJ software. Results are presented as % of control ± S.E.M. and are averaged from 2-4 independent experiments. The statistical significance was calculated using the Mann-Whitney test (n.s., $p>0.05$).

Chapter 4
DISCUSSION & GENERAL CONCLUSIONS

More than two decades ago, ghrelin was first reported as a regulator of growth hormone release and, subsequently as an appetite-stimulating hormone. Ghrelin exerts its effects through the binding to the GHS-R1a, which is expressed in several regions of the body, including the brain. The localization of the GHS-R1a in the hippocampus suggests that ghrelin is involved in hippocampal-dependent functions and, therefore indicates, that this hormone may be a link between metabolism and cognition. This hypothesis was tested more than 10 years ago, when Carlini and colleagues (2002) showed for the first time that the administration of ghrelin in animals led to an increase in memory retention as assessed by behavioural tests. Moreover, Diano et al. (2006) showed that ghrelin administration affected the number of spine synapses in the hippocampus and performance in cognition tests. This evidence proposes a function for ghrelin in modulating hippocampal spines and hippocampal-dependent functions, including cognition. Furthermore, in our lab, Ribeiro et al. (2014) found that about 36% of the GHS-R1a colocalizes with synaptic proteins of glutamatergic synapses in primary hippocampal neurons, therefore suggesting that the GHS-R1a is present in excitatory synapses and may be involved in the modulation of excitatory transmission in the hippocampus. Indeed, using two different synaptic plasticity models to assess the cellular mechanisms underlying the ghrelin effects on hippocampal-dependent function, Ribeiro et al. (2014) showed that ghrelin enhanced LTP expression in CA1 pyramidal neurons of organotypic hippocampal slices, and increased the synaptic accumulation of GluA1 triggered by chemical LTP in cultured hippocampal neurons. These were the first insights into the cellular and molecular mechanisms that may mediate the effect of ghrelin in hippocampal-dependent cognition, and suggest a possible link between the regulation of energy metabolism and learning (Ribeiro et al., 2014).

Most excitatory synapses occur in specific protrusions that come from the dendrites, the dendritic spines or spines. Microstructural changes in dendritic spines are accompanied by changes in synaptic function and dysfunction (Yuste, 2013). Moreover, the changes in dendritic spines depend on synaptic activity and can be modulated by sensory experience. Having this into consideration, it is reasonable to think that spine features or properties may be tightly associated with synaptic function or plasticity. Given the functional relevance of spine structure, and in the context of ghrelin, it has been hypothesized that ghrelin may affect the hippocampus through microstructural changes at the level of dendritic spines (Diano et al, 2006; Berrout et al., 2012). However, the role for ghrelin in hippocampal structural dynamics and plasticity had never been addressed. Having all of this into consideration, our lab has focused on exploring the role for ghrelin in hippocampal spine dynamics. This was the main aim of this thesis project.

Our results suggested that short-term activation of GHS-R1a (1 h) affects hippocampal spine dynamics mainly by favouring an enlargement of small dendritic spines. To further understand the mechanisms involved in these observations, we studied cofilin phosphorylation, implicated in the regulation of actin, and we observed an increase in its phosphorylation upon GHS-R1a activation, enabling us to speculate that the microstructural changes induced by ghrelin can be downstream events of the phosphorylation of cofilin.

Characterization of dendritic spine dynamics in hippocampal slices

To critically analyse and understand the effect of a certain treatment in a model of study, it is of high importance to have a clear understanding of that model, which is, in our case, the organotypic hippocampal slice cultures. Some studies have reported changes in size and shape of spines during development (mainly in primary cultures) but a clear characterization of spines for different developmental stages in organotypic hippocampal slices cultures is lacking in the literature.

The characterization of dendritic spine morphology in organotypic hippocampal slices at different developmental stages showed that the spine density and the spine head diameter from spines on dendrites of CA1 pyramidal neurons are not different between the different studied ages. We also observed that, regardless of the age of the culture, the majority of spines were mature (thin, mushroom and stubby). These results are in line with previous observations showing that some features of the overall population of dendritic spines in a neuron may be preserved over development (Zuo, Yang et al. 2005). Interestingly, the number of filopodia, which are highly dynamic structures, (Matsuzaki, Honkura et al. 2004, Bourne and Harris 2008) decreased and the number of mushroom spines increased as the cultures aged, which is in agreement with the concept that the structural plasticity is reduced during development. However, these observations are not enough to conclude that dendritic spines are not dynamic, since we observed that that proportion of the different types of spine morphologies changes between the different developmental stages of the cultures studied.

Nevertheless, it is important to keep in mind the limitations of our experimental approach, which does not follow single spines over time, rather registering changes in the overall population of spines. One way to study spine dynamics at the single spine level is through a time-lapse experiment. Ideally, we would like to perform these analyses on a single spine of

a given dendrite during the different developmental ages but this would be a demanding experiment, probably impossible given the resources available.

However, it is yet possible to perform a characterization of the spine dynamics in hippocampal slices on a single spine level if we shorten the duration of the experiment. For these experiments we decided to follow the changes in volume of the head of individual spines, every 10 min for a total of 80min experiment, in order to be able to capture more subtle changes. Our observations showed that the overall dendritic spine volume did not suffer changes over the time of the experiment, but there is a large fraction of spines that either shrink (~50%) or grow (~30%) throughout the course of the experiment, which shows that spines behave dynamically. Moreover, this result shows the relevance and robustness of this experimental approach. Changes in the dendritic spines that were not initially detected when considering the pool of all spines were now captured, when focusing in changes of single spine volume over time.

Effects of the GHS-R1a activation on spine dynamics in hippocampal slices

In nature, food sources are not always accessible and animals can go through long periods of time without eating. So, the ability to find food and to remember where food exists is crucial to survive. Therefore, learning and memory are essential processes for survival mainly during periods of food shortage. Ghrelin hormone regulates several processes such as appetite, food intake and also memory, providing a link between metabolic needs and memory. In our lab we have found that ghrelin affects AMPA receptor traffic and synaptic plasticity (Ribeiro, Catarino et al. 2014). Furthermore, we, as others (Diano, Farr et al. 2006, Berrout and Isokawa 2009) observed that ghrelin may have an effect in spine dynamics, but as the literature is very scarce in this subject, we decided to further explore the effects of ghrelin on hippocampal dendritic spines. Ghrelin exerts its effects by binding to its receptor, which expression is increased during development in cultured hippocampal neurons (Ribeiro et al, 2014). It is possible that the distribution pattern of expression of the GHS-R1a on synapses during development is correlated to its effects on the structural level but so far this has not been investigated. Having this into consideration, we sought to investigate if the GHS-R1a activation affects spine morphology and density at different developmental stages.

In this part of the work, we implemented two different paradigms: 1) to mimic fasting, we activated the GHS-R1a for a long period of time (20 h); 2) to make a parallel to the peak of

ghrelin observed prior to a feeding event (Cummings et al., 2001), we activated the GHS-R1a for a short period of time (50 min).

Our results showed that the agonist of the receptor, when incubated with the hippocampal slices for 20h, did not affect spine morphology or density, contrarily to what was observed *in vivo* by Diano et al. (2006), who reported that after a twice a day intraperitoneal administration of ghrelin for 4 days, a much more chronic approach than ours, spine density was increased in the hippocampus. The fact that with our stimulus we did not observe an effect al the level of spine morphology/density does not mean that spines are not dynamic or that ghrelin is not interfering with that dynamics, because we can have groups of spines behaving differently (shrinking or growing) when submitted to the same stimulus in a way that when considering the entire pool of spines, an effect is not observed. With this type of approach, only robust alterations in spine dynamics could be captured. To overcome this issue, we used a time-lapse experiment to study each spine individually previously and after activation of the GHS-R1a with the agonist.

Previous results from our lab showed that when the GHS-R1a is activated during 1h, there is an increase in GluA1, VGlut1, PSD95 and the total area of F-actin clusters in mature hippocampal neurons, as assessed by imunocytochemistry (Fiona van Lewmeen, 2013). Again, as described above, we followed the changes in spine head volume of individual spines every 10min, 30min prior the stimulus with the GHS-R1a agonist and 50min after. Our results showed that the activation of the GHS-R1a with the agonist progressively increased the overall spine head volume, reaching statistical significance after 50min of incubation. Moreover, upon 50 min incubation, we observed a decrease in the number of spines that shrink, an increase in the number of spines that grow and a decrease in the number of spines that do not shrink nor grow, when comparing to the control. It is plausible to think that this effect is partially due to a shift from the populations of spines that shrink (there are less spines shrinking upon incubation with the agonist) to the population of spines that grow (there are more spines growing upon incubation with the agonist). We also observed that the GHS-R1a activation reduces the population of spines do not shrink or grow after 50 min. If we consider that this is the population of stable spines, our results suggest that the agonist decreases the stability of spines. Having in mind studies reporting that not all the spines respond the same way to the same stimulus (Matsuzaki, Honkura et al. 2004), we also wondered whether there is a specific subpopulation of spines that contributes more to the effects herein observed. Our analyses suggest that the agonist affects mainly spines with smaller heads (smaller than the median value of spine head volumes). Furthermore, small

spines from CA1 neurons from slices incubated with the agonist for 30 min showed a significant increase in spine head volume in comparison to the control. When looking at large spines in the control condition, they were significantly reduced in volume after the 80 min of the time-lapse experiment, an effect that was not observed in the presence of the GHS-R1a agonist. This is a very interesting observation as it establishes a duality for the GHS-R1a function at the level of spine dynamics. On one hand, the GHS-R1a increases the volume of small spines, but on other hand it prevents the decrease in volume of large spines. In terms of the proportion of spines that shrink, grow or do not shrink or grow, our data indicates that the GHS-R1a activation affects a much higher number of spines that we could initially think, as the majority of spines either shrunk or grew after the 50 min of incubation with the agonist, while a really small proportion of spines remains unchanged. When comparing these effects considering the two groups of spines, small or large, we see that in both groups there are less spines shrinking and and more spines growing when the GHS-R1a is activated, but in the large spine group the percentage of spines that grow is much higher. This is an interesting observation, since the large spines group did not show an overall volume change when stimulated with the GHS-R1a agonist in comparison to the control. Furthermore, concerning the number of spines that did not shrink or grow, in the small spines group it was the same as in the control condition, whereas in the large spines group, the number in that population was decreased when compared to the control. Considering the two types of evidence together, averaged volume change and proportion change in different populations of spines, it seems that a bigger number of larger spines are responding to the stimulus, but the total volume change that it accounts for is smaller than in the case of small spines. Furthermore, this effect seems to be in close relation to the effect on the reduction on the stable spines observed in this large spines group. We can speculate that the large spines are presumably the more mature spines and therefore less plastic, with a limited capacity to grow. On the other hand, although currently, there is no published evidence describing the localization of the GHS-R1a in the different types of spines, it is possible to speculate that if larger spines present a bigger surface area, they may be expressing more GHS-R1a units than smaller spines (as it is the case for AMPAR, for example), which might account for the effect on the percentage of spines responding to the stimulus.

The different approaches in this part of the work were completely novel to the field of ghrelin effects in hippocampal spine dynamics. We showed that a long-term stimulus does not affect robustly the overall hippocampal spine morphology, but when the neurons are stimulated for a short-time period and each spine is followed individually, we are able to detect microstructural changes favouring an increase in spine volume.

Molecular mechanisms for GHS-R1a effects on spine dynamics in hippocampal slices

Spine dynamics is highly dependent on the spine actin cytoskeleton; therefore, alterations in actin dynamics can underlie changes at the level of dendritic spines. Cofilin is an F-actin-severing protein, which increases F-actin turnover by creating new barbed ends for F-actin growth. In particular, cofilin-mediated spine plasticity is related to spine enlargement during LTP and spine shrinkage during LTD (Bosch et al.(2014) showed that cofilin phosphorylation at Ser-3 induces spine enlargement probably because the affinity of phosphorylated cofilin to actin is decreased, therefore enabling its polymerization into filaments. On the other hand, several studies have reported the involvement of the PI3 Kinase pathway, upstream of the phosphorylation of cofilin, in the formation of dendritic spines (Lee et al, 2011, Kumar et al, 2005). Taking together these evidences and the fact that the PI3 kinase pathway is activated upon GHS-R1a activation (Luis Ribeiro PhD Thesis, unpublished work) we sought to investigate whether cofilin could be involved in ghrelin effects on spine dynamics.

Indeed, our results showed that the GHS-R1a activation may affect the number, area of phosphorylated cofilin clusters in primary cultures and expression of phosphorylated cofilin in both primary and organotypical hippocampal cultures upon different periods of incubation with its agonist. In primary cultures, the expression of phosphorylated cofilin was only statistically significant after 5 hours of incubation with agonist, when considering total levels of p-cofilin. Aditionally, in organotypical slices, although we did not achieve statistical significance, the higher levels of p-cofilin observed between the studied time points are also present at 5 hours. Surprisingly, at 5 hours, also the levels of total cofillin start to increase. These are very interesting effects since it was previously shown (Luis Ribeiro PhD Thesis, unpublished work) that the GHS-R1a activation for 5 hours activates the PKC signalling pathway which was correlated with phosphorylation of Ser816 and Ser818 in the GluA1 C-terminus. Altogether, these results may indicate that the PKC signalling pathway or a related signaling pathway could be involved in both the posttranslational modifications of cofilin and in the synthesis of new cofilin molecules after 5h of incubation with its agonist.

Moreover, the number and the area of the p-cofilin clusters at a total level are very high after only 1h of the GHS-R1a activation. These results can be correlated to an increase in the overall spine head volume observed after 50 min of the GHS-R1a activation, which suggest that the two effects may be related. As described in the previous section, when considering the small spines group, there was a significant increase in spine head volume after

incubation with the GHS-R1a agonist for 30 min, in comparison to the control. Putting this together with the fact that Ribeiro et al. (2014) observed an increase in the levels of phosphorylation of Akt at Ser473, a residue targeted by a PI3 kinase downstream signalling, we can speculate that the structural mechanisms involved in the GHS-R1a activation after 30 min incubation with the agonist may be correlated or dependent on this pathway and may justify the effects we observe at the phosphorylation levels of cofilin in later time points (namely, at 1 hour stimulation). The study of the mechanistic behind the effects of ghrelin in spine dynamics is still very preliminary and in the future we aim to pinpoint the exact role of cofilin phosphorylation in this effect and to specifically target the pathways that seem to be involved in that phosphorylation.

Chapter 5
FUTURE PERSPECTIVES

The work in this thesis showed for the first time that the GHS-R1a activation induces the enlargement of dendritic spines, in a manner that is dependent on the initial spine size. Whereas activation of the GHS-R1a lead to an increase on small spine volume during the 50 min of the experiment, it prevented the shrinkage of larger spines. These observations pose several questions, and open new perspectives to study the role of GHS-R1as in the hippocampus. First, it is important to understand the precise role for the GHS-R1a activation in the spine volume changes observed. What is the expression patter of the GHS-R1a in spines of different volume or shape? How does this correlate with the structural changes observed in this thesis? Are the differential spine responses dependent on their instrinsic characteristics (e.g. smaller spines are more plastic), or on the presence and content of the GHS-R1a? One way to address these questions would be by co-transfecting *mCherry* with a construct for GFP-tagged GHS-R1a in organotypic hippocampal slices, in which we would analyse spine volume changes in response to GHS-R1a activation, in a time-lapse experiment similar to the one we did above, and correlate changes in volume to the presence and abundance of the GHS-R1a. Given that GHS-R1a endocytosis is known to be triggered by its activation, a superecliptic pHluorin (SEP)-tagged form of the GHS-R1a, which allows visualization of the receptor at the cell surface, would be useful to follow the GHS-R1a traffic throughout the experiment. One other aspect that needs to be addressed is the relationship between the functional and structural impact of activation of the GHS-R1a in the hippocampus. GHS-R1a activation triggers the synaptic incorporation of the AMPA receptors (Ribeiro et al., 2014), and we now found that it leads to spine enlargement. It is possible that the structural changes observed are secondary to the functional changes on the level of AMPA receptors, or that the two occur independently. One hypothesis would be that the activation of the GHS-R1a enhances delivery of AMPA receptors at the synapses, together with the membrane from the exocytic vesicles. This would not only enlarge dendritic spines but also incorporate more AMPA receptors at the synapse. To assess this hypothesis, we could co-transfect *mCherry* with the SEP-GluA1 construct, which enables the detection of GluA1 at the surface of the synapses. Following changes in spine volume in parallel with changes in the surface insertion of GluA1 triggered by activation of the GHS-R1a will allow comparing temporal aspects of the two processes.

Altogether, these two groups of experiments would give insights on: 1) how the distribution of the GHS-R1a would correlate with the shape and volume of spines; 2) how spines with different levels of expression of the GHS-R1a respond to its activation; 3) whether volume changes in spines in response to activation of the GHS-R1a are paralleled with spine insertion of AMPA receptors.

Activity-dependent changes in synaptic strength and structure underlie the basis for learning and memory formation. It is tempting to speculate that the spine volume changes triggered by activation of the GHS-R1a that we report here will make spines more prone to undergo structural plasticity in response to activity. Indeed, ghrelin acts in the hippocampus and it has been shown to facilitate LTP expression (Ribeiro et al., 2014). It is possible that it also facilitates structural plasticity. To address this question, we propose to transfect organotypic hippocampal slices with mCherry expressing plasmid and perform chemical LTP or glutamate uncaging to trigger structural plasticity, in control conditions or after activation of the GHS-R1a. Chemical LTD protocols (NMDA receptor-dependent of mGluR-dependent), or low frequency glutamate uncaging can be used to induce spine shrinkage. The single-spine induction of plasticity using glutamate uncaging (either LTP or LTD) is a more subtle strategy to evaluate the effects of GHS-R1a activation. On one hand, this approach is more physiological; on the other hand, with the single-spine induction of plasticity it will be possible to test whether upon activation of the GHS-R1a neighbouring spines can respond to plasticity induced at a single spine, i.e., whether heterosynaptic plasticity can be triggered by activation of the GHS-R1a.

The mechanisms underlying the GHS-R1a activation-mediated changes in spine volume are not clear. We show that the levels of cofilin phosphorylation increase when the GHS-R1a is activated. To further confirm the involvement of cofilin in the structural effect of ghrelin it will be interesting to interfere with cofilin levels, either by overexpressing it or its phosphorylation mutants (cofilin S3A or cofilin S3D) or knocking down its levels and check whether this affects ghrelin effects on spine dynamics.

Taking into account previous data from our lab that showed that the GHS-R1a activation is involved in the activation of the PI3K and the PKC pathways, and knowing that these are upstream modulators of cofilin phosphorylation, it would be interesting to assess the role of the different pathways in the microstructural effects mediated by the GHS-R1a activation. We could incubate primary cultures with inhibitors of these pathways for short periods of time, after activation of the GHS-R1a; and correlate with spine structure, with AMPA receptors surface expression and with P-cofilin levels. Overall, the present work shows that activation of the GHS-R1a in the hippocampus impacts spine dynamics. This is a novel observation that prompts the study of how GHS-R1a activation affects structural plasticity in the hippocampus, and of the relevance of these processes for ghrelin-induced memory enhancement.

Chapter 6
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