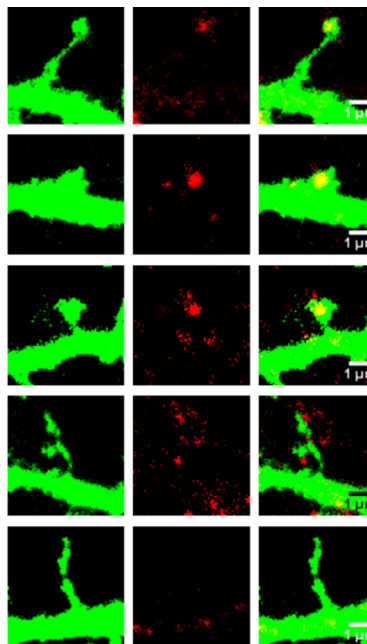# DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Effects of ghrelin on hippocampal glutamate receptors and neuronal morphology

Joni van Leeuwen

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Master in Cellular and Molecular Biology (specialization in Neurobiology)

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Contents

Abreviations

α-MSH: alpha-Melanocyte-stimulating hormone

2-AG: 2 Arachidonoylglycerol

ABP: AMPAR binding protein

ACTH: Adrenocorticotropic hormone

AgRP: Agouti-related protein

Ala: Alanine

AMP: Adenosine monophosphate

AMPAR: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors

AMPK: AMP-activated protein kinase

Arg: Arginine

ARP2/3: Actin-Related Proteins

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

CA: *Cornu ammonis*

Ca^{2+}: Calcium

CaMK: Ca^{2+}/calmodulin-dependent protein kinase

CB1R: Cannabinoid receptor type 1

CBP: CREB-binding protein

CCK: Cholecystokinine

Cdc42: Cell division cycle 42

cGKII: cGMP-dependent kinase II

cGMP: Cyclic guanosine monophosphate

CREB: Cyclic AMP response element-binding protein

CRH: Corticotropin-releasing hormone

DAG: Diacylglycerol

DG: Dentate gyrus

DIV: Days *in vitro*

EPSC: Excitatory postsynaptic current

ERK3: Extracellular signal-regulated kinase 3

GABA: gamma-Aminobutyric acid

GEF: GTP exchange factor

GH: Growth hormone

GHS: Growth hormone secretagogue

GHS-R: Growth hormone secretagogue receptor

Glu: Glutamic acid

GPCR: G protein coupled receptor

GRIP: Glutamate receptor interacting protein

GRs: Glucocorticoid receptors

GTP: Guanosine triphosphate

HEK-293 : Human Embryonic Kidney 293 cells

ICV : Intracerebroventricular

IGF:1: Insulin like growth factor 1

IP3: Inositol-3-phopshate

KCl[:] Potassium chloride

kDa: kiloDalton

Leu: Leucine

LTD: Long-term depression

LTP: Long-term potentiation

MAP: Microtubule-associated protein

MCR: Melanocortin receptors

MEK: Mitogen-activated protein kinase kinase

mEPSCs: Miniature excitatory post-synaptic currents

Mg^{2+}: Magnesium

MK5: Mitogen-activated protein kinase-activated protein kinase 5

MRI: Magnetic resonance imaging

mRNA: messenger RNA

MRs: Mineralocorticoid receptors

mTOR: mammalian target of rapamycin

Na^{+}: Sodium

Ngn2: Neurogenin 2

NMDAR: *N*-methyl-D-aspartate receptor

NO: Nitric oxide

NOS: Nitric oxide synthase

NPY: Neuropeptide Y

NSF: N-ethylmaleimide-sensitive factor

N-WASP: Neural Wiskott–Aldrich syndrome protein

PDZ: Postsynaptic density 95/disc large/zonula occludens 1

Phe: Phenylalanine

Pi3K: Phosphatidylinositide 3-kinase

PICK1: Protein interacting with C-kinase-1

PIP3: Phosphatidylinositol (3,4,5):triphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PKMζ: Protein Kinase Mζ

POMC: Proopiomelanocortin

PSD:95: Postsynaptic density protein of 95 kDa

PYY: Peptide YY

Q: Glutamine

R: Arginine

Rap1: Ras-proximate-1 or Ras-related protein 1

RNA: Ribonucleic acid

S: Serine

Sept7: Septin 7

SER: Smooth endoplasmic reticulum

SERT: Serotonin transporter

Shc : SRC-homology-2-domain-containing transforming protein

siRNA: Small interfering RNA

SRE: Serum response element

SSRI: Selective serotonin re-uptake inhibitors or serotonin-specific reuptake inhibitor

TARPs: Transmembrane AMPAR regulatory proteins

TM: Transmenbrane domain

TrkB: Receptor tyrosine kinases

Trp: Tryptophan

VTA: Ventral tegmental area

WAVE: WASP-family verprolin-homologous protein

Wnt-2: Wingless-type MMTV integration site family member 2

Abstract

Ghrelin is a 28 amino acid peptide hormone produced mainly in the stomach, but also intestine, hypothalamus and pituitary gland. It is an orexigenic substance, meaning it stimulates appetite, and a regulator of growth hormone release. Ghrelin is an endogenous ligand for GHS-R1a (growth hormone secretagogue receptor 1a). This receptor is widely distributed throughout the body, including the hypophysis, hypothalamus and hippocampus. Ghrelin enters the hippocampus, enhancing hippocampal-dependent memory processes. It has been shown to enhance long term potentiation (LTP) and promote spine formation. Thus, ghrelin may represent a molecular link between learning capabilities associated to feeding behavior and energy metabolism, ensuring the ability to locate food sources and remember those locations. Furthermore, GHS-R1a expression pattern seems to be regulated during development in the hippocampus, suggesting that it may play a role in hippocampal development.

Here we show that GHS-R1a expression suffers a consistent increase during development in cultured hippocampal neurons, and that knockdown of the receptor expression decreases arborization complexity in young hippocampal neurons, strongly suggesting that GHS-R1a plays a role in dendritic arborization during neuronal development. Also, neuronal stimulation with the ghrelin receptor agonist MK-0677 caused an increase in dendritic filopodia formation, and a decrease in some types of more mature spines, whereas knockdown of the receptor caused the opposite effect and a decrease in expression of synaptic proteins. These results suggest that both the ligand-mediated and constitutive activity of the receptor are important in spine formation and maturation during development.

In more mature neurons, we show that the ghrelin receptor activation causes an increase in the expression of synaptic proteins, while pharmacological inhibition of the constitutive activity of GHS-R1a decreases the clustering of the synaptic proteins PSD-95 and Vglut. Furthermore, the ghrelin receptor agonist increases F-actin dendritic clustering, suggesting spine enlargement/maturation and/or formation. These results indicate that both the agonist-induced and constitutive activity of the receptor are important in spine formation and/or maturation in mature neurons.

We also observed that the agonist caused an increase in GluA1 surface expression in hippocampal slices, suggesting a role for ghrelin in priming AMPARs for synaptic incorporation, a probable mechanism through which it enhances LTP.

Altogether our results indicate an important role for ghrelin and its receptor in regulating morphological and functional aspects of excitatory synapses in the hippocampus.

Keywords: Ghrelin, GHS-R1a, hippocampus, spine formation, dendritic arborization.

Resumo

A grelina é uma hormona peptídica composta por 28 aminoácidos produzida principalmente pelo estômago mas também pelo intestino, hipotálamo e hipófise. É uma substância que estimula o apetite, e que regula a secreção da hormona de crescimento. A grelina é um ligando endógeno de GHS-R1a (receptor 1a dos secretagogos da hormona de crescimento). Este receptor distribui-se largamente pelo organismo, incluindo a hipófise, o hipotálamo e o hipocampo.

A grelina entra no hipocampo e aumenta processos de memória dependentes do hipocampo. Foi demonstrado que a grelina aumenta a potenciação de longa duração (LTP) e promove a formação de espículas. Assim sendo, a grelina pode representar um elo molecular entre as capacidades de aprendizagem associadas ao comportamento alimentar e o metabolismo energético, assegurando a capacidade para localizar fontes de alimento e recordar esses locais. Além disso, o padrão de expressão do GHS-R1a parece ser regulado no hipocampo durante o desenvolvimento, sugerindo que pode desempenhar um papel no desenvolvimento do hipocampo.

Neste trabalho demonstramos que a expressão do GHS-R1a sofre um aumento consistente durante o desenvolvimento de neurónios do hipocampo, e que uma diminuição da expressão do receptor diminui a complexidade da arborização em neurónios jovens, o que sugerefortemente que o GHS-R1a tem um papel na arborização dendrítica durante o desenvolvimento neuronal. Além disso, a estimulação com o agonista MK-0677do receptor da grelina causa um aumento na formação de filopódios e uma diminuição nalguns tipos de espículas mais maduras enquanto a diminuição da expressão do recetor provoca o efeito oposto e uma diminuição na expressão de proteínas sinápticas. Estes resultados sugerem que quer a atividade mediada pelo ligando quer a atividade constitutiva do recetor da grelina são importantes na formação e maturação de espículas durante o desenvolvimento.

Em neurónios mais maduros, demonstrámos que o agonista do receptor da grelina causa um aumento na expressão de proteínas sinápticas, enquanto a inibição farmacológica da atividade constitutiva do GHS-R1a causa uma diminuição da aglomeração das proteínas sinápticas PSD-95 e Vglut. Além disso, o agonista causa um aumento na aglomeração dendrítica de F-actina, o que sugere um aumento/maturação e/ou formação de espículas. Estes resultados indicam que tanto a atividade induzida por agonista como a atividade constitutiva do recetor são importantes na formação e/ou maturação de espículas nos neurónios maduros.

Também observámos que o agonista provoca um aumento da expressão de GluA1 superficial em fatias de hipocampo, sugerindo que a grelina desempenha um papel na incorporação sináptica dos recetores AMPA, um mecanismo provável através do qual a grelina aumenta o LTP.

No seu conjunto, os nossos resultados indicam um papel importante para a grelina e o seu receptor na regulação de aspetos morfológicos e funcionais de sinapses excitatórias no hipocampo.


Palavras-chave: Grelina,GHS-R1a, hipocampo. formação de espiculas, arborização dendrítica.

1. INTRODUCTION

1.1 The hippocampus

The hippocampus is a structure in the brain that is involved in episodic memory and spatial memory (reviewed in S.-H. Wang & Morris 2010). It comprises three primary regions: the dentate gyrus (DG), the CA3 (*cornu ammonis* 3) region and the CA1 region. Axons of dentate granule cells (granule cells in the DG), the mossy fibers, project onto the pyramidal cells of the CA3 region. These neurons, in turn, send axons (the Schaffer collaterals) to the pyramidal cells of the CA1 region (fig. 1) (reviewed in Kullmann & Lamsa 2007).

The hippocampus connects to many other structures in the brain, such as the thalamus, the hypothalamus, the basal forebrain, the entorhinal cortex and the amygdala (reviewed in Bird & Burgess 2008).
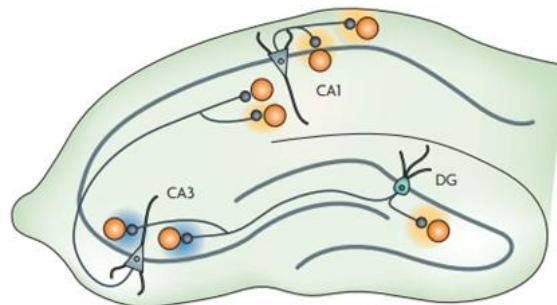
Figure 1: The main regions and pathways in the hippocampus. CA1: CA1 region, CA3: CA3 region, DG: dentate gyrus. (Adapted from Kullmann & Lamsa 2007).

1.1.1 Glutamatergic synapses

The primary type of excitatory synapse in the hippocampus is the glutamatergic synapse. Glutamatergic terminals characteristically communicate with dendritic spines, which have an actin-based cytoskeleton that serves as an anchor for cytoplasmic and membrane proteins (reviewed in Derkach et al. 2007); they contain many proteins, making up the so-called postsynaptic density. Proteins present in spines include glutamate receptors, scaffold proteins such as postsynaptic-density protein of 95 kDa (PSD-95), signaling proteins such as Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) (reviewed in Li & Sheng 2003) and in many cases, smooth endoplasmic reticulum (SER) (Yuste 2010) (fig. 2).

Dendritic spines

Spines can greatly vary in shape: they can be stubby, thin, mushroom-shaped or branched (ramified) (fig. 3) (Arellano et al. 2007). Stubby spines are devoid of a neck, and are particularly prominent during early postnatal development, though they are also found in the adult (Yuste 2010). The typical thin spine has a long neck and a small, bulbous head (Yuste 2010), and mushroom spines have a large head (Yuste 2010) and usually contain have a higher content of glutamate receptors and the most complex postsynaptic densities (reviewed in Bourne & Harris 2008). They are typically found in adult samples (Yuste 2010). Branched spines represent a small but significant proportion of spines (Yuste 2010). No cases have been reported where the two heads of such a spine make

synapses with the same axon (Yuste 2010). In addition to the various types of spines, there are filopodia, developmentally transient structures (Yuste 2010) thought to give rise to spines, which are long, thin and often branching (Yuste 2010) protrusions that lack a clear head and the typical postsynaptic density of mature spines (Yuste 2010).
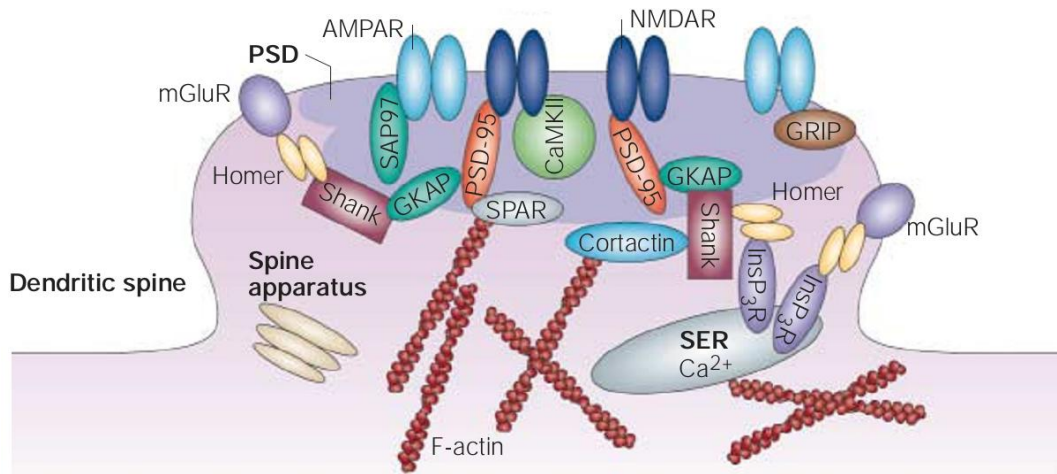
Figure 2: Schematic respresentation of a dendritic spine and its molecular components (Adapted from Z. Li & Sheng 2003)
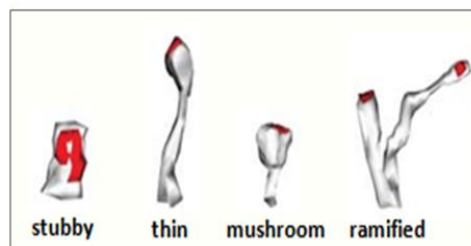
Figure 3: Typical morphologies of each type of spine (stubby, thin, mushroom and ramified (Adapted from Arellano et al. 2007)

The thin spine neck hinders Ca^{2+} exchange between the spine and the dendritic shaft. Therefore, spines with a thin neck and a large head are more efficient in confining Ca^{2+} transients compared to spines with a thick neck and a small head. This ability of dendritic spines to compartmentalize Ca^{2+} from the dendritic shaft and other spines may be a key feature of their function and also enable synapse-specific plasticity (further discussed below) (reviewed in Ethell & Pasquale 2005).

Filopodia sometimes transform into more stable thin or mushroom spines (reviewed in Bhatt et al. 2009). Mushroom spines tend to be more stable than thin spines (reviewed in Harms & Dunaevsky 2007). Thin spines have been proposed to be "learning spines" that turn into mushroom spines (proposed to be "memory spines") upon synaptic potentiation (reviewed in J. Bourne & Harris 2007). The role of stubby spines in neuronal function is still controversial.

During development, there is spine formation but also spine elimination or "pruning" (reviewed in Bhatt et al. 2009 and Penzes et al. 2011). For example, in young adolescent mice, sensory whisker experience appears to cause spine elimination in an *N*-methyl-D-aspartate receptor (NMDAR)-dependent manner (Zuo et al. 2005).

Increases in spine number have been reported with various learning paradigms, including motor skill training and spatial memory (reviewed in Harms & Dunaevsky 2007). On the other hand, a number of psychiatric and neurological diseases are associated with alterations in spine morphology

or density (reviwed in Bhatt et al. 2009). Down's syndrome is associated with decreased spine density in the neocortex and hippocampus, and schizophrenia with decreased spine density in the neocortex. In Alzheimer's disease, the best correlate of cognitive dysfunction is thought to be the loss of synapses and in fragile X syndrome, which is the most frequent form of inheritable mental retardation, spines are found in much higher density and display a more immature, long and thin form. Abnormal spine morphology and number also occur in disorders such as depression or addiction (reviewed in Bhatt et al. 2009). MRI studies reveal progressive grey-matter loss before and during psychosis development in schizophrenia in late adolescence, suggesting synaptic over-pruning (reviewed in Jan & Jan, 2010).

The formation and maturation of spines, as well as their structural plasticity at mature synapses, depend on actin filament dynamics (Ethell & Pasquale 2005; Sekino et al. 2007). In rat hippocampal slice cultures, there seems to be more spine turnover at young ages (11 DIV(days *in vitro*)) than older ages (24 DIV), i.e. there is more spine formation, more spine elimination and more changes in spine morphology (De Roo et al. 2008). The proportions of filopodia, mushroom and stubby spines, however, did not significantly differ between these two ages, but the authors did show that enlargement of the spine head was part of the maturation process and made spines more stable (De Roo et al. 2008) Stabilization coincided with the formation of PSD-95 and blockade of synaptic activity decreased stabilization and the formation of PSD-95, suggesting that PSD-95 plays a role in activity-dependent spine stabilization (De Roo et al. 2008). In cultured rat hippocampal neurons, spine morphology appears to be developmentally regulated: while at 7 DIV, neurons contain mostly filopodia, at 18 DIV the number of mushroom spines is increased (Sala et al. 2001). In fact, in cultured rat hippocampal neurons 17-21 DIV of age, about 60% of spines on pyramidal cells are mushroom spines (Pak et al. 2001).

Septins (a group of GTP-binding proteins) are also important in spine formation and morphology. Sept7 (septin 7), for example, plays a role in spine formation and morphogenesis, and depends on the ERK3/MK5 (Extracellular signal‑regulated kinase 3/ Mitogen-activated protein kinase-activated protein kinase 5) module, which interacts with Sept7 (Brand et al. 2012). This pathway also promotes dendrite development.

Brain-derived neurotrophic factor (BDNF) increased spine density in CA1 pyramidal neurons (Tyler & Pozzo-Miller 2003), and also plays a role in dendritic arborization.

Glutamate receptors

Glutamate receptors, present in spines, can be ionotropic or metabotropic. Ionotropic receptors are cation channels whose opening is enhanced upon glutamate binding (Siegel et al. 2006). Metabotropic receptors activate intracellular enzymes through G proteins, modulating synaptic transmission (Siegel et al. 2006).

In mammals there are four major families of ionotropic glutamate receptors: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs), kainate receptors, NMDARs and delta receptors (Smart & Paoletti 2012). The excitatory postsynaptic current (EPSC) is typically mediated by AMPARs and NMDARs.

Kainate receptors are found pre- and postsynaptically and have a modulatory role (reviewed Lerma 2006 and Contractor et al. 2011).

Delta receptors are the least understood and are apparently incapable of gating an ion channel following ligand binding, making them electrically "silent" (Kohda et al. 2000; Schmid et al. 2009).

NMDARs subunits are composed of four GluN1-GluN3 subunits: they require two GluN1 subunits and either two GluN2 subunits or a combination of GluN2 and GluN3 subunits (reviewed in Traynelis et al. 2010). NMDARs are permeable to Ca^{2+} and Na^+ (Siegel et al. 2006) and require glycine as a co-agonist (reviewed in Meldrum 2000). They are blocked by Mg^{2+}, though this can be overcome by depolarization (reviewed in Meldrum 2000). This means that NMDARs conduct currents only when glutamate is bound and the postsynaptic neuron is depolarized, so pre -and postsynaptic neurons need to be active simultaneously to open NMDARs.

AMPARs are heterotetramers made of at least two of their subunits GluA1-GluA4 (fig. 4) (Nakagawa 2010). However, when overexpressed, an individual AMPAR subunit is capable of assembling into functional homomeric receptors (Nakagawa 2010). GluA1, GluA4 and an alternative splice form of GluA2 (GluA2L) have long cytoplasmic tails (fig. 4). GluA3, the predominant splice form of GluA2 and an alternative splice form of GluA4 (GluA4S) have short cytoplasmic tails (fig. 4) (reviewed in Shepherd & R. L. Huganir 2007). In rat hippocampus, AMPARs are normally composed of GluA1 and GluA2 subunits or GluA2 and GluA3 subunits (R. Wenthold et al. 1996), while GluR4 is mainly expressed early in development (R. Wenthold et al. 1996). The GluA2/GluA3 receptors are continuously recycled at the plasma membrane and the GluA1/GluA2 receptors are inserted during synaptic plasticity (Shi et al. 2001). AMPARs mediate fast excitatory glutamatergic neurotransmission. They are permeable to Na^+ and, only in some cases, to Ca^{2+} (Siegel, G., Alberts, R.W., Brady, S., Price 2006). GluA2 subunits usually suffer RNA editing at a specific site, where the glutamine (Q)-encoding codon is converted to an arginine (R)-encoding codon (fig. 4). AMPARs that contain Q have high permeability to Ca^{2+} and are sensitive to polyamine channel blockers, whereas AMPARs containing R have low Ca^{2+} permeability and are insensitive to polyamine channel blockers (Traynelis et al. 2010).

The AMPARs subunits have postsynaptic density 95/disc large/zonula occludens 1 (PDZ) consensus motives in their intracellular C-terminal regions, that interact with several PDZ domain–containing proteins (reviewed in H.-J. Lee & Zheng 2010 and Henley 2003). PKC (protein kinase C) phosphorylates GluA2 in one of these motifs, in serine 880 (S-880) (fig. 4), through which GluA2 binds to glutamate receptor interacting protein (GRIP), AMPAR binding protein (ABP) and protein interacting with C-kinase-1 (PICK1) (Chung et al. 2000; Matsuda et al. 2000; Matsuda et al. 1999; Seidenman et al. 2003; Perez et al. 2001). This phosphorylation prevents the association of GluA2 with GRIP and promotes binding to PICK1 (Perez et al. 2001; Matsuda et al. 1999; Chung et al. 2000), thus recruiting it to synapses, and facilitating internalization of surface receptors (Matsuda et al. 2000). Although PICK1, GRIP1 and ABP are evidently important in AMPAR trafficking, their precise role is not yet clear. AMPARs have many other phosphorylation sites and binding partners (fig. 4), as will be further discussed in the next section.

1.1.2 Molecular mechanisms of learning and memory

The two major forms of long-lasting synaptic plasticity in the mammalian brain are long-term potentiation (LTP) and long-term depression (LTD) (reviewed in Collingridge et al. 2010). LTP is characterized by a long-lasting increase in synaptic strength and LTD by a long-lasting decrease in synaptic strength (reviewed in Collingridge et al. 2010), and both appear to be important for learning and memory (reviewed in Collingridge et al. 2010).

Various forms of LTD have been described, of which the most studied is NMDAR-dependent LTD, usually induced by low frequency stimulation (reviewed in Collingridge et al. 2010). Most synapses that undergo LTD use L-glutamate as their neurotransmitter, and in LTD they lose sensitivity to glutamate, probably through removal of AMPARs from the synapse (reviewed in Collingridge et al. 2010).

LTP occurs when NMDARs are activated, leading to Ca^{2+} influx (reviewed in Minichiello 2009). In order for NMDAR channels to open, both sufficient membrane depolarization (which expels the Mg^{2+} block from NMDAR channels) and L‑glutamate binding are required (reviewed in Minichiello 2009). Ca^{2+} directly or indirectly triggers the activation of several enzymes that mediate LTP (reviewed in Minichiello 2009) (fig. 5).

While the first phase of LTP (early LTP), occurring in the first 60–90 minutes, is thought to be mediated primarily by protein phosphorylation and delivery of new receptors to the postsynaptic sites, the maintenance of LTP (late LTP), occurring thereafter, is dependent on new gene transcription and mRNA translation, leading to the stabilization of existing synapses and the formation of new synapses (reviewed in Derkach et al. 2007).
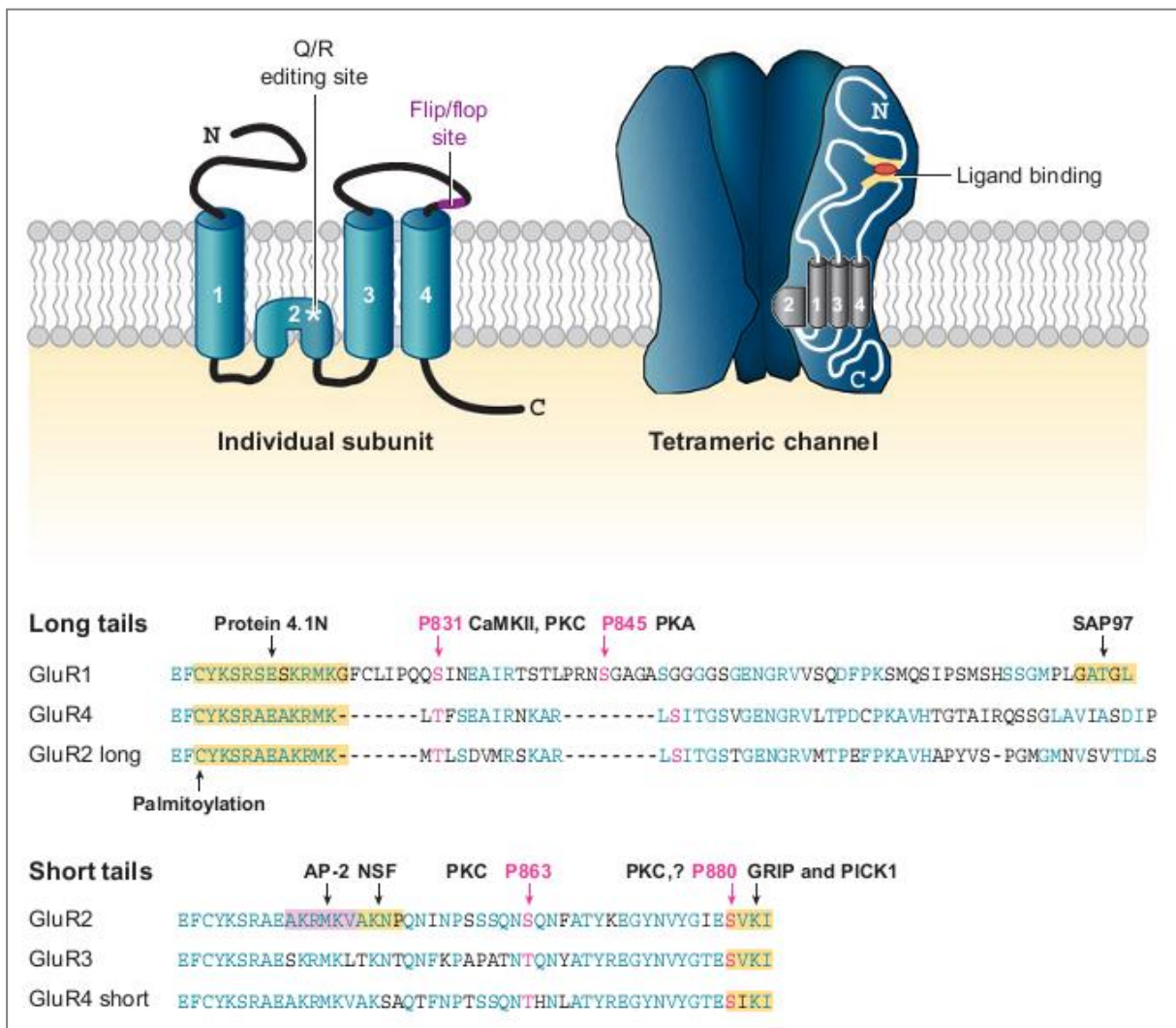
Figure 4: Structure and composition of AMPARs. Top: Structure of the AMPAR subunits and the tetrameric channel. The individual subunits are composed of four transmembrane domains (the Q/R-editing site is shown, in the 2nd transmembrane domain), and the channel consists of four subunits, which are usually two dimers. The dimers are usually two different subunits. **Bottom:** Subunits with long cytoplasmic tails and subunits with short

cytoplasmic tails. Some of the binding partners (arrows) and phosphorylation sites and respective protein kinases on different subunits are shown. (Adapted from Shepherd et al, 2007).

Early LTP

One of the main events during LTP consists on the phosphorylation of various serine residues on GluA1 subunits: Serine 831 (S-831) can be phosphorylated by protein kinase C and CaMKII (figs. 4 and 5), serine 845 (S-845) can be phosphorylated by protein kinase A (PKA) (figs. 4 and 5) and cGMP-dependent kinase II (cGKII), and serine 818 (S-818) and 816 (S-816) are phosphorylated by PKC (reviewed in Santos et al. 2008; Lin et al. 2009). The Ras–MEK–ERK (Ras - Mitogen-activated protein kinase kinase -ERK) and Ras–PI3K–Akt (Ras - Phosphatidylinositide 3-kinase - Akt) pathways are also required to induce GluA1-mediated synaptic potentiation, and they seem to mediate phosphorylation of S-845 and S-831, respectively (Qin et al. 2005).
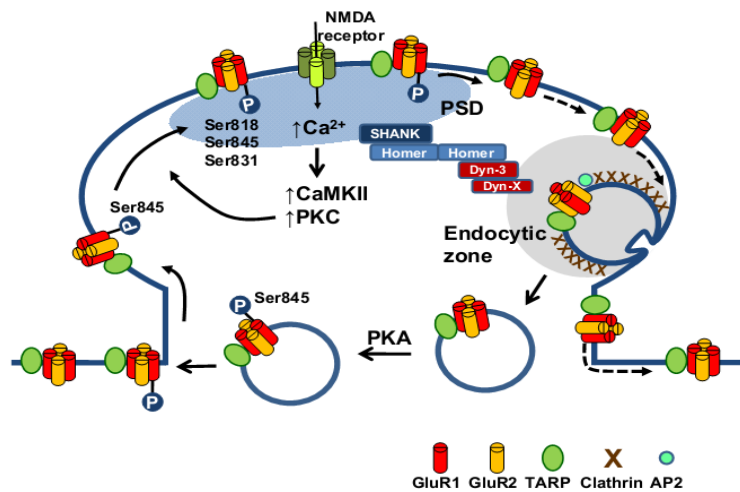
Figure 5: Trafficking of GluA1-containing AMPARs. Ca^{2+} influx caused by NMDAR activation leads to the activation of various enzymes, such as CaMKII and PKC, which, among other functions, phosphorylate serine residues on GluA1 subunits, thus playing a role in their synaptic incorporation. S-845 is phosphorylated by PKA, which is necessary for AMPAR synaptic incorporation (Adapted from Santos et al, 2008).

S-818 and S-816 phosphorylation enhances binding of 4.1N protein (fig. 4) to GluA1 (Lin et al. 2009). 4.1N is an actin-binding protein required for activity-dependent GluA1 insertion, which binds GluA1, stabilizing its surface expression (Shen et al. 2000; Lin et al. 2009).

Both S-831 and S-845 phosphorylation occur upon induction of LTP in the CA1 region of the hippocampus (reviewed in Santos et al. 2008). Mice with S-831 and S-845 mutated to alanine show reduced LTP in the CA1 region of hippocampus, as well as defective spatial memory as assessed by the Morris water maze test (Lee et al. 2003), indicating that phosphorylation of the GluA1 subunit is required for LTP and spatial memory formation. Phosphorylation of S-831 increases channel conductance and drives synaptic delivery of AMPARs (fig. 5), and phosphorylation of S-845 increases their channel open-probability and is necessary for their synaptic incorporation (fig. 5) (Gomes et al. 2003). Synaptic GluA1 insertion leads to increased synaptic strength by potentiating glutamate response (reviewed in Santos et al. 2008). BDNF has been shown to induce GluA1 phosphorylation on S-831 through activation of PKC and CaMKII and promote synaptic delivery of homomeric GluA1 AMPARs (Caldeira et al. 2007). S-831 phosphorylation does not seem to be required for receptor synaptic delivery, however, since mutation of S-831 to alanine, which prevents its phosphorylation by CaMKII, doesn't prevent delivery of receptors to synapses by CaMKII (Hayashi 2000). Oh and coworkers saw that receptors phosphorylated at S-845 were trafficked specifically to extrasynaptic

sites but not to synapses; however, these extrasynaptic AMPARs can be incorporated into synapses by NMDARs activation (Oh et al. 2006). Thus, they proposed that S-845 phosphorylation primes AMPARs for synaptic potentiation by trafficking them to extrasynaptic sites, possibly followed by synaptic incorporation requiring synaptic activity (Oh et al. 2006). Mutating a predicted PDZ domain interaction site in GluA1 C-terminus blocked the CaMKII-dependent synaptic delivery of GluA1 (Hayashi, 2000), suggesting that CaMKII-dependent phosphorylation of an unidentified substrate containing a PDZ domain is required for GluA1 synaptic clustering.

Transmembrane AMPAR regulatory proteins (TARPs), that bind AMPARs, cluster them at the synapse through their interaction with PSD-95 (reviewed in Santos et al. 2008). Stargazin is a TARP phosphorylated by CaMKII and PKC (reviewed in Santos et al. 2008), and phosphorylation facilitates its binding to PSD-95, required for synaptic clustering of AMPARs (Tomita et al. 2005). In hippocampal neurons, CaMKII activation (through NMDARs) stops the diffusion of surface AMPARs at synaptic sites by stargazin phosphorylation and binding to PSD-95, thus indirectly stabilizing AMPARs at synapses (Opazo et al. 2010). PI3K was found to be required for AMPA receptor insertion during LTP (Man et al. 2003). It is responsible for a constant supply of PIP_3 necessary to ensure PSD-95-mediated clustering of AMPARs at the postsynaptic membrane (Arendt et al. 2010).

Another event that occurs during LTP is an increase of F-actin content within spines (Fukazawa et al. 2003), leading an increase in spine-size (fig. 6) (reviewed in Lin et al. 2004). This probably functions to allow for the reorganization of the PSD and the incorporation of the AMPA receptors (reviewed in Lin et al. 2004); indeed, actin filaments in spines regulate the molecular organization of the postsynaptic density (Kuriu et al. 2006). A widening and shortening of the spine neck has also been reported to follow LTP (fig. 6) (reviewed in Lamprecht & LeDoux 2004). There is a correlation between spine size and synaptic strength (Derkach et al. 2007), and changes in dendritic geometry during plasticity also seem to affect propagation (Vetter et al. 2001). It has been shown that activation of NMDA receptors in hippocampal neurons leads to an increase in spine size within 5 minutes (reviewed in Lin et al. 2004).
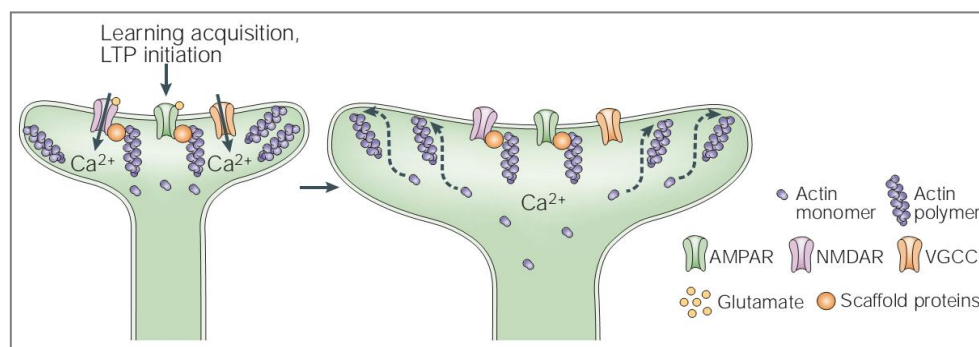
Figure 6: An increase in spine size, mediated by an increase in F-actin content, and an increase in spine neck width rapidly follow LTP induction (Adapted from Lamprecht and LeDoux, 2007)

Late LTP

The persistence of LTP for hours or days (late LTP) is thought to depend on local dendritic protein synthesis and nuclear transcription (Minichiello, 2009). PKC (Mao et al. 2007), PKA, Ca^{2+}/calmodulin dependent protein kinase IV (CaMKIV) and ERK lead to the activation of transcription factors such as

cyclic AMP‑responsive element‑binding protein (CREB protein) (Minichiello, 2009), which promotes synthesis of other transcriptional regulators, protein kinases, AMPARs, etc. (Purves et al. 2008). Protein kinase Mζ (PKMζ), a constitutively active isoform of PKC, is necessary for maintenance of late-LTP and several forms of long-term memory (reviewed in Sacktor, 2011). Translation of its mRNA can be induced during LTP (reviewed in Sacktor, 2011), and it maintains the increased number of synaptic AMPAR by enhancing the ability of NSF (N-ethylmaleimide-sensitive factor) to release GluA2-containing receptors from PICK1 (reviewed in Sacktor, 2011). Inhibition of PKMζ reverses LTP maintenance *in vivo* and produces persistent loss of 1-day-old spatial memory, indicating a possible distinction between early LTP as a learning substrate, and late LTP as a memory substrate (Pastalkova et al. 2006).

LTP induction in the dentate gyrus of freely moving adult animals increases the content of polymerized actin (F-actin) in spines in the hippocampus, and these elevated F-actin levels persist for at least five weeks after stimulation. Blockade of actin polymerization in adult rats prevents the development of late-phase LTP (8 h), leaving the initial amplitude and early phase (30–50 min) of LTP intact (reviewed in Lamprecht & LeDoux 2004), suggesting that actin dynamics contribute to the maintenance of LTP. The endurance of spine enlargement (induced by polymerization of actin) is dependent on CaMKII (Honkura et al. 2008), as is the outgrowth of new spines (reviewed in Von Bohlen & Halbach, 2009), which is also induced by LTP (reviewed in Fischer et al. 2000). LTP induction in the dentate gyrus also caused an increase in the number of branched spines (Yuste, 2010). The orientation, kinetics of assembly and stability of F-actin filaments are known to contribute to spine shape and are regulated by extracellular stimulation that could contribute to spine formation and changes in spine shape after LTP (for example, NMDAR activation) (reviewed in Lamprecht & LeDoux 2004)**.** Inhibition of NMDARs blocks LTP and active polymerization of actin (reviewed in Lamprecht & LeDoux 2004). So NMDAR-dependent actin polymerization appears to be important for the consolidation of the early phase of LTP into the late phase in adult rats *in vivo*. (reviewed in Lamprecht & LeDoux 2004).

Functional expression of AMPA receptors is dependent on F-actin (Honkura et al. 2008), probably because AMPARs are indirectly linked to the actin cytoskeleton. Shank3, a postsynaptic scaffold protein that indirectly links membranar proteins such as AMPARs and NMDARs to the actin cytoskeleton (Phelan & McDermid 2011), increases actin polymerization, thereby promoting spine formation. Shank3 mutations identified in autism spectrum disorder disrupt these processes (Durand et al. 2012).

Activation of NMDA receptors in hippocampal neurons can lead formation of new spines and protrusions after about 20 minutes (reviewed in Lin et al. 2004).

1.1.3 Dendritic arborization

There are several phases in dendritic arbor development (fig. 7). This sequence of events seems to be very similar between species, although the time scale may differ. The (partially overlapping) stages are: neurite initiation, outgrowth and guidance; branching and synapse formation, and stabilization. Initial dendrite growth is relatively slow, but is followed by a period of very fast dendritic extension. Subsequently, dendritic branching occurs, and stabilization of the dendritic arbor occurs over a long period of time. Though during development of dendritic arbor there are high rates

of branch additions and retractions, the mature dendritic arbor is less plastic with a very low branch turnover. Even so, dendritic arbors in the mature nervous system preserve some degree of plasticity. (reviewed in Urbanska et al. 2008).

Dendritic arbor shape is one of the crucial factors determining how signals coming from individual synapses are integrated (reviewed in Urbanska et al. 2008). Exposure to enriched environments or training on a motor-learning task increases dendritic growth and branching in cortical pyramidal neurons (reviewed in Wong & Ghosh, 2002), and spatial learning in rats has been shown to increase the complexity of the dendritic arbor of adult newborn hippocampal neurons (Tronel et al. 2010). Mental disorders such as autism spectrum disorders and Rett's syndrome are often associated with abnormal brain size, suggesting overgrowth or lack of dendrite pruning as well as alterations of neuronal number during development, while schizophrenia could arise from over-pruning or failed maintenance of dendrites later in life (reviewed in Jan & Jan, 2010).
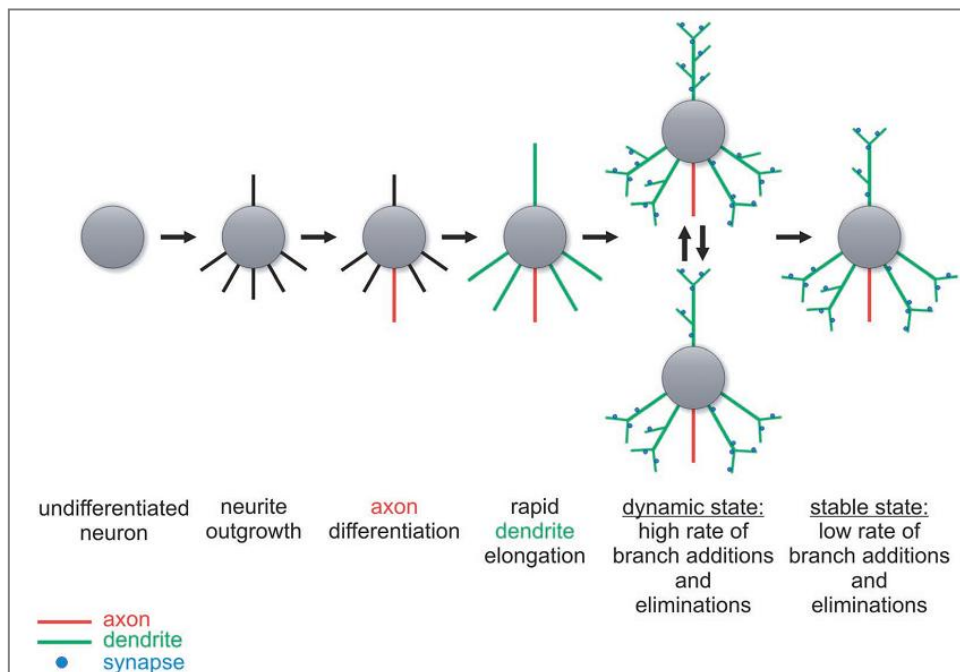
Figure 7: The various (partially overlapping) stages of dendritic arbor development (Adapted from Urbanska et al. 2008).

There are many factors involved in dendrite arborization. Both intrinsic genetic mechanisms and signals from the extracellular environment play a role. The mediators are intracellular processes: signal transduction, cytoskeleton dynamics, transcription, translation, and cellular membrane turnover.

In the intrinsic genetic component, various transcription factors determine dendritic patterning independently from extracellular cues (reviewed in Urbanska et al. 2008). In mammals, neurogenin 2 (Ngn2), for example, defines a specific pattern of dendritic arborization in pyramidal neurons in cerebral cortex (Hand et al. 2005). Extracellular signals affecting dendritic arborization, plasticity and stability include diffusible cues, cell contacts, and neuronal activity. BDNF, for example, has been shown to increase total dendritic length and arbor complexity. BDNF/TrkB receptor signaling, through the adaptor protein Shc, is a crucial pathway for BDNF-dependent dendrite outgrowth in hippocampal neurons. The pathway downstream of Shc includes Ras - ERK, and PI3K - Akt. (Y. Sato et al. 2011).

High levels of corticosterone, via activation of the glucocorticoid receptors, appear to be able to reduce the dendritic complexity of CA1 pyramidal neurons in young, developing rat hippocampal tissue (Alfarez et al. 2009). The effects of neuronal activity on dendritic arborization are widely accepted to be due to elevated cellular concentrations of Ca^{2+} (reviewed in Urbanska et al. 2008). Neuronal transmission can either increase or decrease dendritic arborization: in *Xenopus laevis*, a four-hour light exposure led to an increase in dendrite growth dynamics and total dendritic length, and this effect was blocked by inhibition of the AMPA and NMDA receptor-mediated glutamatergic transmission (Sin et al. 2002). Spatial learning in rats has been shown to increase the complexity of the dendritic arbor of adult newborn hippocampal neurons in an NMDAR-dependent manner (Tronel et al. 2010). On the other hand, GABA-based transmission has been shown to increase dendritic growth of rat young interneurons (Gascon et al. 2006).

Countless intracellular mechanisms regulate dendritic arbor. Proteins that play a major role herein are small G-proteins of the Ras family, protein kinases and protein phosphatases.

Signaling pathways that control dendritic arborization

Ras is an important protein in dendritic branching. It leads to an increase in the total number of dendrites and the dendritic arbor complexity of hippocampal neurons in culture (reviewed in Urbanska et al. 2008), and its effects have been shown to depend on PI3K, extracellular signal-regulated kinases (ERKs), phospholipase D, and mammalian target of rapamycin (mTOR) kinase (reviewed in Urbanska et al. 2008). Other members of the Ras family have also been shown to increase dendritic branching, such as Rap1, which has been suggested to act via ERK kinases and activation of CREB-dependent transcription (Chen et al. 2005).

All major protein kinases known to be active in neurons influence dendritic arbor development. They include CaMK, ERK, PKA, PI3K and receptor tyrosine kinases (TrkB). In *X. laevis,* CaMKII led to premature stabilization of the dendritic arbors in younger neurons (longer branch life time) and a lower net increase of total dendritic length (Wu & Cline 1998). CaMKIIα controls neuronal activity-dependent growth of dendrites in cerebellar neurons by phosphorylation of NeuroD at S336 (Gaudillière et al. 2004). Overexpression of CaMKIIβ was able to bind to and regulate the actin cytoskeleton and increased dendritic arborization in hippocampal neurons (Fink et al. 2003). CaMKIV and CaMKI were shown to control dendritic growth and arborization induced by either BDNF or neuronal activity, and both CaMKs control dendritic arbor growth at a transcriptional level (Redmond et al. 2002; Wayman et al. 2006). PI3K has also been shown to play a major role in dendritic arbor development and stability: overexpression of a constitutively active form of PI3K led to increased dendritic arbor complexity in hippocampal neurons in vitro (Jaworski et al. 2005). Downstream molecular mechanisms of PI3K involved in dendritic arbor development and stability involve both protein synthesis and the regulation of actin cytoskeleton dynamics (reviewed in Urbanska et al. 2008). A constitutively active form of Akt mimicked the dendritic branching effects of increased PI3K activity, and both PI3K-constitutively active- and Akt-constitutively active-dependent dendritic branching were blocked by inhibition of mTOR kinase (a well known regulator of protein synthesis) (Jaworski et al. 2005).

Sept7 stimulates dendritic outgrowth through the ERK3/MK5 complex in primary hippocampal neurons (Brand et al. 2012).

The cytoskeleton in the regulation of dendritic arborization

A tight control of actin and microtubule cytoskeleton organization is essential for the formation of proper cell morphology. Some well known regulators of cytoskeleton dynamics belong to the Rho family of small GTPases, such as RhoA, Rac1 and Cdc42 (cell division cycle 42), and regulation of the actin cytoskeleton during dendritic arborization is dependent on Rho GTPases (reviewed in Urbanska et al. 2008). In most studies published thus far, increased activity of RhoA and inhibition of Rac1 or Cdc42 resulted in significant simplification of the dendritic trees in many neuron types, while activation of Rac1 or Cdc42 caused an increase in the number of dendrite branches (reviewed in Urbanska et al. 2008). Accelerated dendritic arbor development of *Xenopus* optic tectum neurons induced by 4h light exposure has been shown to depend on Rho GTPases, and electrical stimulation of *Xenopus* optic nerve in fact led to increased activity of Rac1 and decreased activity of RhoA, in an NMDAR- and AMPAR-dependent manner (Li et al. 2002). Mammalian Rac1 GEFs Kalirin-7 (Xie et al. 2007) and Tiam-1 (Tolias et al. 2005) have been shown to be necessary for NMDA-induced Rac1 activation, and in fact Kalirin-7 knockdown led to a simplification of hippocampal neuron dendrites (Ma et al. 2003). Rac1 and Cdc42 indirectly activate Arp2/3 (involved in actin polymerization) via WAVE (WASP-family verprolin-homologous protein) and N-WASP (neural Wiskott–Aldrich syndrome protein), respectively (reviewed in Urbanska et al. 2008), and overexpression of N-WASP increased the number of neurites and branch points in developing hippocampal neurons in vitro (Pinyol et al. 2007). PICK1, which binds Arp2/3, disrupts Arp2/3 binding to N-WASP and inhibits Arp2/3-mediated actin polymerization (reviewed in Urbanska et al. 2008). PICK-1 knockdown caused increases in proximal dendrite branching and decreases in distal arborization (Rocca et al. 2008). Cypin binds to tubulin heterodimers and promotes the assembly of microtubules, and RhoA decreases its levels, resulting in decreased dendrite number. Several non-Rho GTPase-related microtubule binding proteins regulate the development and stability of dendritic arbor morphology, like MAPs (microtubule-associated proteins), microtubule plus-end tracking proteins, and motor proteins. Phosphorylation MAP2, a microtubule-stabilizing protein, by different kinases can exert opposite effects on dendritic arborization (reviewed in Urbanska et al. 2008).

The role of transcription and translation in the regulation of dendritic arborization

Transcription plays an essential role in dendritic arborization, both in the intrinsic genetic mechanism and in activity-dependent dendritic arbor growth. CREB-binding protein (CBP) is a general chromatin modifier and transcription coactivator crucial for CREB transcriptional activity, shown to play a fundamental role in dendritic arborization (reviewed in Urbanska et al. 2008). Inhibition of CBP has been shown to block CaMKIV-dependent and KCl-induced dendritic growth of cortical neurons in vitro (Redmond et al. 2002). Overexpression of various forms of dominant negative mutants of CREB or siRNA also prevented KCl-, CaMKIV- and CaMKI-induced dendritic growth in cortical and hippocampal neurons (Redmond et al. 2002; Wayman et al. 2006), while overexpression of a constitutively active CREB mutant was sufficient to increase total dendritic length (Wayman et al. 2006). Wnt-2 (wingless-type MMTV integration site family member 2), expression of which depends on CREB, is an important gene for activity-driven dendritic growth (Wayman et al. 2006).

Dendritic arbor development also requires protein translation. Several proteins important for control of dendritic branching, like CaMKII, glutamate receptor subunits, BDNF, TrkB, GRIP1 and PSD-

95, are translated locally in dendrites, often in a neurotrophic factor-, neuronal activity- and mTOR-dependent fashion (reviewed in Urbanska et al. 2008).

Finally, membrane trafficking plays a crucial role in dendritic growth, which requires constant additions of membrane to the cell surface (reviewed in Urbanska et al. 2008).

But dendritic arborization is not yet fully understood: just one example of a remaining question concerns the exact molecular differences between development, stabilization and further remodeling of dendritic arbors.

1.2 Regulation of food intake

In order to avoid mal-nutrition and obesity, energy intake must approximately match energy expenditure. The body is able to regulate food ingestion through tightly regulated physiological mechanisms, and the hypothalamus is essential for this control. It receives many signals in this respect, such as neural signals that present sensory information about the fullness of the stomach, chemical signals from circulating nutrients, hormonal signals, etc.

Substances that regulate appetite can be divided into orexigenic substances, that stimulate feeding, and anorexigenic substances, that inhibit feeding (Guyton & Hall, 2006). They include hormones such as leptin, cholecystokinine (CCK), insulin, ghrelin, peptide YY (PYY) and glucocorticoids, and neuropeptides like orexins and neuropeptide Y (NPY). Leptin is released into the blood by adipose tissue in proportion to the abundance of this tissue and leads to a decrease in food intake and a decrease in insulin secretion. CCK is released in the small intestine when fat enters the duodenum and promotes satiety, decreasing food intake. Insulin also decreases food intake and is secreted by the pancreatic Islets of Langerhans in response to an increase in the level of glucose in the blood. Ghrelin, on the other hand, acts to increase food intake. It is secreted by the stomach between meals, when the stomach is empty (Guyton & Hall, 2006). PYY is secreted specially from the ileum and colon in response to food intake (Guyton & Hall, 2006) and suppresses appetite (Fox, S. I., 2003). Orexins are, as the name suggests, orexigenic, as are glucocorticoids (Guyton & Hall, 2006), which can inhibit insulin secretion (Lambillotte et al. 1997).

Stimulation of the lateral nuclei of the hypothalamus causes animals to increase feeding, while stimulation of its ventromedial nuclei causes satiety, believed to be mediated by inhibition of the lateral nuclei. Certain neurons in the arcuate nuclei of the hypothalamus receive signals from various hormones involved in food intake and energy expenditure: the proopiomelanocortin (POMC) neurons and the neurons that produce NPY and agouti-related protein (AGRP) (NPY-AGRP neurons) (fig. 8). Their location, close to fenestrated capillaries at the base of the hypothalamus, allows for the access of these hormones (Benoit et al. 2000; Cone et al. 2001).
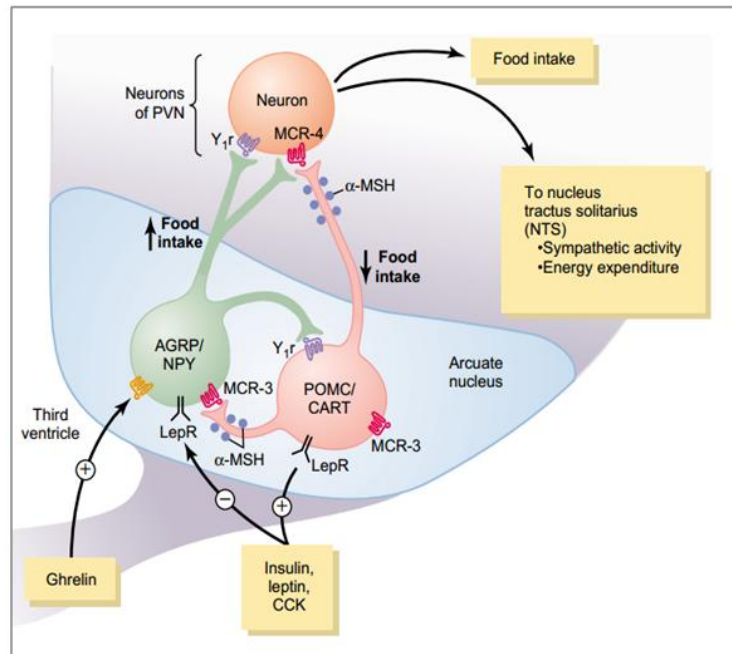
Figure 8: Regulation of food intake by AGRP/NPY and POMC neurons in the hypothalamus (Adapted from Guyton & Hall, 2006).

POMC neurons are stimulated by leptin, CCK, insulin (Guyton & Hall, 2006) and PYY (Fox, 2003). They release α-melanocyte-stimulating hormone (α-MSH, a derivate of POMC) which then activates melanocortin receptors such as MCR-3 and MCR-4 in the paraventricular nuclei of the hypothalamus, leading to a decrease in food intake and an increase in energy expenditure. This seems to be mediated, at least in part, by stimulation of sympathetic nervous system activity. NPY-AGRP neurons are stimulated by ghrelin, and inhibited by leptin, cholecystokinin (CCK), and insulin. When stimulated they release AGRP, which is a MCR-3 and MCR-4 antagonist, which acts to inhibit the actions of α-MSH. These GABAergic neurons also release NPY, which stimulates appetite, when energy stores are low. (Guyton & Hall, 2006). Leptin (Guyton & Hall, 2006)and PYY (Fox, 2003) decrease NPY production and glucocorticoids stimulate NPY production, at least in part by inhibiting mTOR signaling in the hypothalamus (Shimizu et al. 2010). In addition, NPY-AGRP neurons can inhibit POMC neurons by GABA release (reviewed in Gao and Horvath, 2007). So, when activated, the NPY-AGRP neurons lead to an increase of food intake and a decrease in energy expenditure (Guyton & Hall, 2006).

Other areas in the hypothalamus innervated by NPY/AGRP and POMC neurons are the lateral hypothalamic area (which contains neurons that produce the orexigenic neuropeptides melanin concentrating hormone and orexins), the dorsomedial nucleus and the ventromedial nucleus (Simpson et al. 2009).

1.3 Cognitive effects of hormones

Some metabolic hormones and stress hormones have also been shown to exert effects on brain structures involved in memory, such as the hippocampus, amygdala and prefrontal cortex.

Corticosterone, the principal glucocorticoid in rodents (in humans, it is cortisol), slowly increases upon stress and activates mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). These receptors are present in regions that are critical for memory formation, such as the hippocampus, the amygdala and the prefrontal cortex. MRs are already activated at low levels of corticosterone, while GRs are only activated at high levels. Initial glucocorticoid binding to MRs in hippocampus increases the release probability of glutamate, the lateral diffusion of AMPARs, the activity‑dependent synaptic insertion of AMPARs, enhancing LTP, and the frequency of mEPSCs (miniature excitatory post-synaptic currents). The latter effect has also been observed in the amygdala. These effects are mediated by non‑genomic events and might be relevant for the formation of memories related to a stressful event. Posterior activation of GRs leads to long-lasting genomic effects (prolonged until after glucocorticoid levels return to normal) that although causing increased synaptic incorporation and lateral diffusion of GluA2‑containing AMPARs, suppress the ability to induce LTP, and facilitate LTD (by facilitating the endocytosis of GluA2 subunits). Thus, glucocorticoids can both promote and impair LTP, and this dual role might serve to reinforce memory of a stressful event and suppress memory formation of non-relevant events that occur after the stressful event. (reviewed in Krugers et al. 2010).

Noradrenaline and CRH (corticotropin-releasing hormone), also involved in stress response, promote synaptic plasticity and noradrenaline has actually been shown to promote emotional learning through GluA1-containing AMPARs. (reviewed in Krugers et al. 2010).

Leptin promotes the induction of hippocampal LTP through the facilitation of NMDA receptor function (reviewed in Harvey 2007). It has been shown to promote the synaptic expression of GluA1, and cause an enhancement of EPSCs, maintained for up to 90 min (Moult et al. 2010). Furthermore, it increases the density and motility of dendritic filopodia through actin reorganization (an event coupled to development and synaptic plasticity) and, subsequently, the density of hippocampal synapses. These events appear to be dependent on synaptic activation of NR2A-containing NMDA receptors and mediated by the ERK signaling pathway. (O'Malley et al. 2007). Direct administration of leptin into the hippocampus has been shown to improve learning and memory in rats. (Moult et al. 2010). Also, it seems to bring on a novel form of hippocampal LTD, induced under conditions of increased excitability and negatively regulated by PI3-kinase. (Moult et al. 2010). Interestingly, insulin also causes hippocampal LTD, wherein PI3K and PKC have been implicated. Some studies suggest the involvement of GluA2 containing AMPAR tyrosine phosphorylation and internalization. (Moult et al. 2010).Insulin also increases hippocampal neurons' surface expression of GluA1 subunits and NMDARs and enhances their NMDA receptor mediated currents. In addition, it increases the expression of PSD-95 in the CA1 area in rat hippocampal slices. (Moult et al. 2010). Intracerebroventricular administration of insulin has been shown to improve performance in an avoidance memory task in rats, and intrahippocampal administration improved spatial memory. Blockade of endogenous hippocampal insulin, using antibody-like peptides, impairs memory. (McNay 2007).

Insulin like growth factor 1 (IGF-1), another anabolic hormone, increases dendritic branching in cortical pyramidal neurons and IGF-1 knockout animals exhibit a reduction in spine density (Moult et al. 2010). A positive correlation between IGF-I levels and cognitive function has been found in healthy elderly people and in elderly individuals with mild cognitive impairment. (reviewed in Van Dam et al. 2000).

Ghrelin can also function as a cognitive enhancer and hippocampal modulator, as will be further discussed below.

1.4 Ghrelin

Ghrelin is a 28 amino acid hunger-stimulating peptide hormone (Gualillo et al. 2003) (fig. 9) that is an endogenous ligand for growth hormone secretagogue receptor (GHS-R) (Kojima et al. 1999). Its discovery started with the discovery of growth hormone secretagogues (GHS), now classified as ghrelin mimetics. GHSs are synthetic compounds that are potent stimulators of GH (growth hormone) release, now known to work through the GHS-R (Howard et al. 1996; Korbonits et al. 2004; Pong et al. 1996). This receptor was cloned in 1996, (Howard et al. 1996), and remained an orphan GPCR (a GPCR with no known natural bioactive ligand) until 1999, when the endogenous ligand ghrelin was finally purified and identified) (Kojima et al. 1999).

Acylation of serine-3 (Ser-3) with octanoate (fig. 9) is necessary for its activity and for binding to its receptor, growth hormone secretagogue receptor 1a (GHS-R1a) (Kojima & Kangawa 2005) (Kojima et al. 1999). Ser-3 is conserved in mammals, birds, and fish (Kojima & Kangawa 2005), and octanoylation of ghrelin has been conserved in vertebrates over millions of years of evolution (Kojima & Kangawa 2005; reviewed in Sato et al. 2012). GHS-R1a has also been highly conserved in vertebrates (Yang et al. 2008).
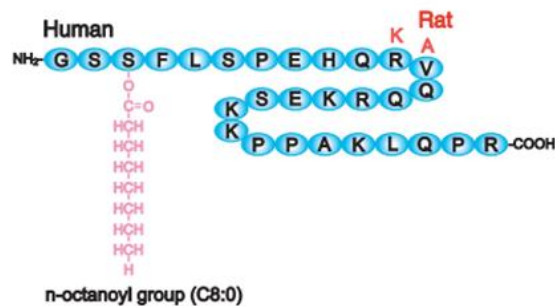
Figure 9: Human and rat ghrelin. Rat ghrelin differs from human ghrelin by only 2 aminoacids. Both forms acylated at serine 3. (Adapted from Kojima & Kangawa, 2005)

Ghrelin is expressed in X/A-like cells in rodents (P/D1 cells in humans) in the oxyntic glands, is secreted into circulation (Inui et al. 2004) and is able to cross the blood-brain barrier (BBB) (Banks et al. 2002). Although its main production site is the stomach, some ghrelin is produced in the intestine, and many other sites of expression, such as the hypothalamus and the pituitary gland, have been identified (Korbonits et al. 2004). Ghrelin- producing neurons are present in the arcuate nucleus and paraventricular nucleus (Kojima & Kangawa 2005). Although human ghrelin can cross the BBB in both the blood-to-brain and the brain-to-blood direction, very little mouse ghrelin seems to cross in the blood-to-brain direction (Banks et al. 2002). Obesity and old age decrease the transport of human ghrelin across the blood-brain barrier, while fasting tends to enhance it (Banks et al. 2008). Apart from GH release and feeding, ghrelin has many other roles in the body, such as in stress, reward and cognition (discussed below).

The ghrelin gene initially gives rise to prepro-ghrelin, which goes through a series of modifications before reaching its mature form ghrelin (Garg 2007). Proteolytic cleavage of pro-ghrelin can also originate obestatin (Garg 2007), a 23-amino acid appetite-suppressing peptide (Zhang et al. 2005). In rat and mouse there are two types of ghrelin precursors: the 117 -amino acid prepro-ghrelin (Kojima

et al. 1999), which contains ghrelin, and a 116-amino acid precursor, which contains des-Gln-14-ghrelin. Gln-14-ghrelin is acylated in a similar manner to ghrelin (Kojima et al. 1999), a modification necessary for its activity. It is able to activate GHS-R1a and induce intracellular Ca^{2+} concentration increases in cells expressing this receptor, and when intravenously injected into rats, it increases the GH concentration is plasma, similarly to ghrelin injection (Hosoda et al. 2000). The ratios observed between prepro-ghrelin and prepro-des-Gln14-ghrelin was 5 to 1 in rat stomach, and 6 to 5 in mouse stomach (Hosoda et al. 2000; Tanaka et al. 2001).

There is some evidence that the catabolic hormone glucagon may increase the expression of ghrelin (Wei et al. 2005). Other important determinants of ghrelin secretion are glucose, insulin, leptin, somatostatin, growth hormone, thyroid hormones, melatonin, and the parasympathetic nervous system, (Korbonits et al. 2004; Yin et al. 2009).

GHS-R1a (ghrelin receptor), is the only receptor specific for acylated ghrelin that has been identified so far (Nakahara et al. 2010). This receptor is widely distributed throughout the body, present in adipose tissue, myocardium, gonads, lungs, liver, arteries, stomach, pancreas, thyroid, etc. (Ferrini et al. 2009). It is also expressed in the pituitary, hypothalamus and hippocampus in both humans and rats (Guan et al. 1997), as well as in other brain regions.

The human ghrelin receptor GHS-R gene encodes for two transcripts: transcript 1a encodes a full-length receptor (GHS-R1a) and transcript 1b codifies for a shortened version (GHS -R1b) (Ploeg & Howard, 1997).

GHS-R1a has seven transmembrane domains (Ferrini et al. 2009) and its C-terminal is in the intracellular region (0 and usually couples to Gq-protein (fig. 10) (Ferrini et al. 2009). Upon ghrelin binding, the Gq-protein activates phospholipase C leading to the production of inositol-3-phopshate (IP3) and diacylglycerol (DAG), leading to the release of Ca^{2+} from intracellular stores and the activation of PKC (Ferrini et al. 2009). PKC inhibits potassium channels through tyrosine phosphorylation, causing further depolarization, which in turn causes the opening of voltage-dependent L-type calcium channels in the plasma membrane, allowing for a more sustained intracellular Ca^{2+} rise. (Korbonits et al. 2004) (Fig. 11). In somatotropic pituitary cells, this depolarization leads to the release of GH (reviewed in Muccioli et al. 2007). Recently it was shown that ghrelin enhances firing of nigral dopaminergic neurons by inhibiting voltage-gated potassium Kv7/KCNQ/M-channels through activation of the PLC-PKC path-way (Shi et al. 2013).
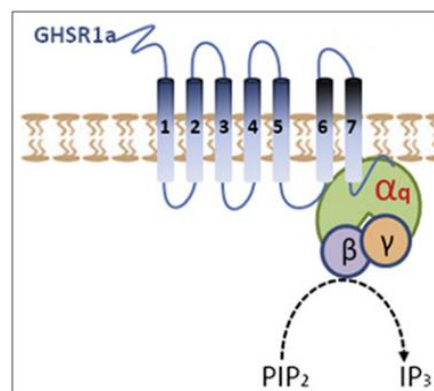
Figure 10: GHS-R1a coupled to Gq protein. The receptor is highly conserved in vertebrates. (Adapted from Schellekens et al, 2010).
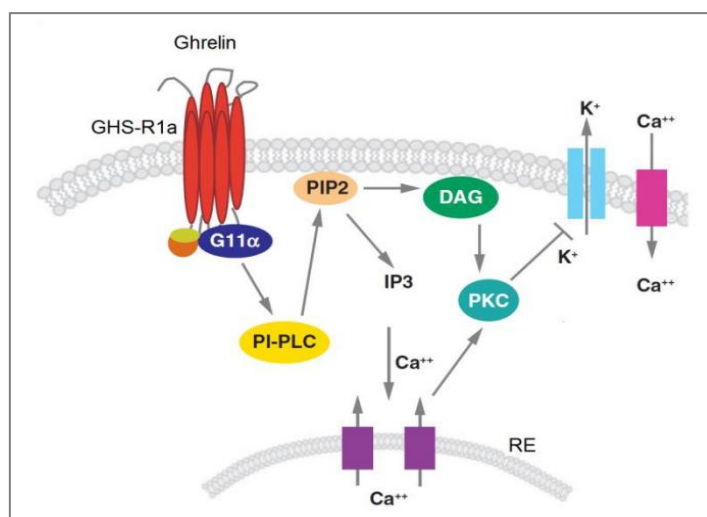
Figure 11: Prototypical signaling of GHS-R1a. Ghrelin binding leads to activation of Gαq/11 protein that activates phosphatidylinositol-specific phospholipase C (PI-PLC), which hydrolyzes phosphatidylinositol 4,5-diphosphate (PIP2), stored in the plasma membrane, to give diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 then causes the release of Ca^{2+} from endoplasmic reticulum, and Ca^{2+}, together with DAG, activates PKC. PKC inhibits K+ (potassium) channels, leading to a depolarization that causes the opening of voltage-dependent L-type Ca2+channels. (Adapted from (Gao and Horvath, 2007).

Another possible pathway is the Gq-CaMKIV- CREB protein pathway: ghrelin has been shown to activate CREB (Holst et al. 2003). Furthermore, ghrelin can activate ERK and PI3K cascades to promote celular proliferation (Kim et al. 2004a, 2004b; Mazzocchi et al. 2004), through various G protein subunits including Gq and a PTX-sensitive G protein (Gi/o) (Camiña, 2006).

There has also been some speculation about the possibility of a switch occurring between Gq and Gs proteins, perhaps cell-type specific: in somatotropic pituitary cells, GHS-R1 a is Gq-coupled, while in neurons it is hypothesized to be Gs coupled (Schellekens et al. 2010). Ghrelin induces Ca^{2+} infux through N-type Ca^{2+} channels in NPY producing hypothalamic neurons. As N-type Ca^{2+}channels are modulated by PKA, this suggests that GHS-R1a is coupled to Gs in these neurons. (Kohno et al. 2003).

In porcine somatotropes (cells in the anterior pituitary that produce growth hormone), ghrelin-stimulated GH secretion depends on activation of nitric oxide/cyclic guanosine monophosphate (cGMP) signaling (Rodríguez-Pacheco et al. 2005). In the hypothalamus, it was found that ghrelin enhances the activity of 5'-AMP-activated protein kinase (AMPK) (Andersson et al. 2004), although the mechanism is still unknown. AMPK is strongly implicated in energy homeostasis and is downregulated by leptin administration in the hypothalamus (Carling, 2005).

Ghrelin has also been shown to a stimulate serum response element (SRE) activity but, here too, the mechanism is not yet clear (Holst et al. 2004).

Apart from its ligand-mediated activity, GHS-R1a also has a high constitutive activity (Holst et al. 2003), which can be demonstrated using an inverse agonist for this receptor (Petersen et al. 2009). The only known inverse agonist for GHS-R1a is (D-Arg1,D-Phe5,D-Trp7,9,Leu11)-substance P (Holst et al. 2003, 2006) This constitutive activity can be mediated through the same pathways: the phospholipase C pathway, the CREB protein pathway, and the SRE pathway (Holst et al. 2004), and it seems to be important for the expression of NPY (believed to mediated by CREB) and food intake (Petersen et al. 2009). The molecular basis of G-protein coupled receptors' (GPCRs) constitutive activity appears to be related with three aromatic residues, namely PheVI:16 (phenylalanine at position 16 in transmembrane domain 6), PheVII:06 and PheVII:09. This region promotes the

formation of a hydrophobic core between helices 6 and 7, to ensure proper docking of the extracellular end of TM7 (transmembrane domain 7) into TM6, mimicking agonist activation and stabilizing the receptor in the active conformation (Holst et al. 2004).

GHS-R1a polymorphisms, Ala204Glu (alanine at the position 204, located in the second extracellular loop, exchanged for a glutamate) and Phe279Leu (phenylalanine at the position 279, located in the TM6, exchanged for a leucine – corresponding to PheVII:06), have been associated with obesity and short stature, respectively (Wang et al. 2004). Phe279 (PheVII:06) has an essential role in holding the ghrelin receptor in the active conformation (Holst et al. 2004) and Ala-204 also seems to play an important role in the constitutive activity, for when GHS-R1a carrying the mutation Ala204Glu is transfected in HEK-293 cells, it displays decreased constitutive activity and lower expression at the plasma membrane (Pantel et al. 2006). Altogether, these observations suggest that selective loss of ghrelin receptor constitutive activity causes a syndrome of short stature and obesity (Holst and Schwartz, 2006).

The constitutive activity of GHS-R1a has also been implicated in the control of food intake and body weight in vivo. The intracerebroventricular injection of the inverse agonist decreased the food intake and body weight in rats. The basal level of CREB phosphorylation in a hypothalamic cell line was decreased by treatment with the inverse agonist and increased by treatment with ghrelin. (Petersen et al. 2009).

GHS-R1b does not have, until now, known biological activity (Howard et al. 1996). However, it has been proposed that this truncated ghrelin receptor may modulate the function of full-length version of the receptor. In fact, GHS-R1a can be retained in the endoplasmic reticulum by heterodimerization with GHS-R1b, consequently decreasing its constitutive activity due to reduced cell surface expression (Chow et al. 2012).

1.4.1 Non-cognitive functions of ghrelin

As mentioned, ghrelin is an orexigenic substance, and its levels increase at night and before meals, and decrease after meals (Yin et al. 2009), in proportion to the amount of calories ingested (reviewed in Chen et al. 2009). Long-term fasting also promotes a rise in ghrelin levels (Yin et al. 2009), as do caloric restriction and chronic stress (Lutter et al. 2008).

Ghrelin induces the release of GH via a dual mechanism involving the hypothalamus and the pituitary. Peripherally or centrally administered ghrelin seems to stimulate the hypothalamic ARC GHS-R as well as the pituitary GHS-R directly to release GH. (Korbonits et al. 2004).

Ghrelin's effects on feeding are independent of its GH-stimulating effect: They are mediated through NPY/AgRP (reviewed in Chen et al. 2009) and POMC (Kojima & Kangawa 2005) neurons in the arcuate nuclei, and orexin-producing neurons in the lateral hypothalamus, and seem to depend on serotonin and cannabinoid signaling (reviewed in Chen et al. 2009). Circulating ghrelin promotes the expression of AgRP and NPY (reviewed in Chen et al. 2009) and it acts on NPY cells axons connecting to POMC cells, inducing the release GABA to inhibit the POMC neurons (reviewed in Gao and Horvath, 2007). It has been suggested that ghrelin-expressing neurons in the hypothalamus also have a important role on regulation of appetite. In the arcuate nucleus, these neurons send efferent fibers onto NPY/AgRP neurons to stimulate the release of these orexigenic peptides, and GABA, which supresses the POMC neurons. (Cowley et al. 2003).

Ghrelin also attenuates glucose-induced insulin release in pancreatic β-cells (reviewed in Chen et al. 2009). It further causes a decrease in energy expenditure, and an increase in body weight and adiposity independent of its effects on food intake (reviewed in Chen et al. 2009). In rodents, ghrelin-induced gain weight is based on accumulation of fat mass by reducing fat utilization (Tschöp et al. 2000). Ghrelin further leads to an increase in gastric acid secretion (Korbonits et al. 2004). Given these important functions, we can easily imagine a relation of ghrelin with conditions like obesity or anorexia nervosa. In fact, GHS-R1a antagonists improved glucose tolerance, suppressed appetite, and caused weight loss in rats, making them promising therapeutic agents for controlling obesity. Patients with anorexia nervosa have increased ghrelin levels, but had a lower GH response to ghrelin administration. Their high ghrelin levels seem to lead to a desensitization of GHS-R1a. (reviewed in Chen et al. 2009).

Feeding elicits rewarding signals, mediated via dopamine release in the mesolimbic circuitry system, which can actually override satiety and stimulate appetite independently of metabolic needs (Kenny, 2011). This is being recognised as a key component in the underlying causes for the increase in obesity incidence (Schellekens et al. 2012). It involves extra-hypothalamic neurocircuitry, including cortical areas as well as areas within the mesolimbic dopaminergic pathway, the ventral tegmental area (VTA), nucleus accumbens, hippocampus and amygdala (Skibicka and Dickson, 2011). Ghrelin has recently emerged as one of the major contributors to reward-driven feeding (Diz-Chaves, 2011; Perelló and Zigman, 2012; Schellekens et al. 2012). Injection of ghrelin in VTA has in fact been shown to stimulate food intake (Naleid et al., 2005; Abizaid et al., 2006).

In the paraventricular nucleus of the hypothalamus, ghrelin stimulates NPY release, which then suppresses GABA release, thereby lifting the inhibition of corticotropin-releasing hormone (CRH)-expressing neurons. These can then release CRH, leading to ACTH and cortisol release (Kojima & Kangawa 2005). It has in fact been shown that intraperitoneal injection of ghrelin elevated serum corticosterone levels in mice (Asakawa et al. 2001). These authors saw that intra-third cerebroventricular ghrelin administration induced anxiogenic behaviour in mice as assessed by entries into the open arms in the elevated plus maze test, and co-injection of a CRH receptor antagonist inhibited these effects (Asakawa et al. 2001). Carlini and coworkers showed that injection of ghrelin into the third ventricle increased freezing in the open-field test in rats, and decreased time spent in the open arms of the plus-maze test, both indexes of anxiety (Carlini et al. 2002). However, both subcutaneous ghrelin injection and calorie-restriction induced ghrelin elevation caused anxiolytic- and antidepressant-like effects in mice, as assessed, respectively, by the elevated plus maze, and the forced swim test (Lutter et al. 2008). Furthermore, chronic social defeat stress (which elevated ghrelin levels) caused greater social avoidance in wild-type that in GHSR$^{-/-}$ mice, indicating that ghrelin may help them cope with stress (Lutter et al. 2008). The reason for the conflicting functions of ghrelin on stress is thus far unclear (Andrews, 2011).

Ghrelin has many additional functions, in processes such as sleep-wake regulation (Steiger et al. 2011), cardiac and gastrointestinal function, reproductive tissue, amog others (Korbonits et al. 2004). It has also been increasingly shown to have cognitive effects, as will be further discussed below.

1.4.2 Cognitive functions of ghrelin

Learning to locate food and remembering those locations are evolutionarily important skills for survival (Moran and Gao, 2006), especially in times of hunger when sources are scarce. This suggests

that these abilities may be tied to signaling molecules involved in energy balance (Moran and Gao, 2006), such as ghrelin, and convincing links between ghrelin and memory have in fact been found.

GHS-R1a expression has been observed in the hippocampus in the CA1, CA2 and CA3 subfields of Ammons's horn and in the dentate gyrus (Diano et al., 2006; Cuellar and Isokawa, 2011; Berrout and Isokawa, 2012; Shi et al. 2013). A large fraction of GHS-R1a seems to localize to excitatory synapses in rat hippocampal neurons (Ribeiro et al., unpublished data). GHS-R1a mRNA is undetectable in the rat fetal hippocampus, but present at high levels at postnatal day 7 (Katayama et al. 2000), suggesting that GHS-R1a expression is regulated during development in the hippocampus.

In 2002 it was shown for the first time, through step-down inhibitory avoidance test, that ghrelin increases memory retention in rats (later shown to be linked to the hippocampus (0 when icv injected immediately after training, suggesting an increase in memory retention (0. Chen and colleagues report that ghrelin is able to activate the PI3K signaling pathway in the hippocampus of rats, accompanied by an enhancement of spatial memory in the Morris water maze test when injected repetitively into the hippocampus a few days before training (0. Furthermore, this peptide was shown to enhance hippocampal-dependent memory as assessed by the novel object recognition test using ghrelin$^{-/-}$ mice, and improve performance in the step-down passive avoidance test when administered immediately after training, suggesting an effect on memory consolidation (Diano et al. 2006). Intra-hippocampal ghrelin injection improved memory retention in the step-down test when administered before the training session, but not when administered before the test session, suggesting that ghrelin could affect processes involved in memory acquisition and/or consolidation, but not in memory retrieval (Carlini et al. 2010). Additionally, in SAMP8 mice (with a phenotype mimicking pathological and cognitive signs of Alzheimer disease), ghrelin improved retention in the T-maze foot shock avoidance test (Diano et al. 2006). Icv (intracerebroventricular) administration of ghrelin, after training, impaired memory performance in the passive avoidance task in neonatal chicks, suggesting a conserved evolutionary role for ghrelin in memory regulation in vertebrates (Carvajal et al. 2009). Ghrelin receptor knockout mice also show impaired performance in the Morris water maze test (Davis et al. 2011).

Ghrelin was found to promote formation of spines on hippocampal dendrites, to increase LTP (0, and to enhance the maintenance of LTP in the DG in a MAP kinase-dependent manner (Chen et al. 2011b). Interestingly, this last effect was only detected in the late-phase of LTP (dependent on protein synthesis), but not in the early phase of LTP (that lasts for up to 2 h and requires modification of pre-existing proteins) (Kelleher et al., 2004; Lynch, 2004). Application of ghrelin for 1 or 23 h in organotypic hippocampal slices increased the phalloidin (mushroom toxin with a high affinity for polymerized F-actin) fluorescence signal in the CA1 region, suggesting an increase in dentritic spine density in this hippocampal region (Berrout and Isokawa, 2012). Cuellar and Isokawa demonstrated in rat hippocampal slices that ghrelin activated CREB through cAMP and PKA, suggesting that in this region the ghrelin receptor may also be coupled to Gs protein. Additionally, this effect of ghrelin was shown to involve NMDARs, and it was blocked by the endocannabinoids anandamide and 2 arachidonoylglycerol (2-AG) [agonists of the type 1 cannabinoid receptor (CB1R), a Gi protein-coupled receptor]. Ghrelin promoted PKA-dependent phosphorylation of the GluN1 subunit of NMDARs (blocked by anandamide and 2-AG), and stimulated F-actin reorganization (phalloidin binding to F-actin), which was suggested to be a consequence of CREB-induced gene expression. (Cuellar and Isokawa, 2011).

Multiple recent evidence from our lab show that ghrelin or ghrelin receptor agonist MK-0677 lead to synaptic incorporation of GluA1 in rat hippocampal neurons and organotypic slices. This was shown to be an activity- and NMDAR-dependent process. MK-0677 application to hippocampal organotypic slices significantly enhanced LTP expression, while having no effect on the non-potentiated pathway. This suggests that GHS-R1a activation facilitates classic NMDAR-dependent LTP. Furthermore, pre-treatment of cultured neurons with MK-0677 for 1 or 20 h before cLTP protocol significantly increased surface and synaptic GluA1 content. Several pathways activated during LTP, known to be involved in synaptic GluA1 incorporation/maintenance, were tested. MK-0677 treatment led to an upregulation of the PKC pathway, with a significant increase after 5 h of treatment, and activation of the PI3-kinase pathway within 30min. GluA1 phosphorylation at S-831 and S-845 were also increased and stargazin phosphorylation at S-239/240 was significantly induced 20 h after the addition of MK-0677 (Ribeiro et al, unpublished data).

It has further been suggested that serotonin reuptake inhibitors (SSRI) have an inhibitory effect upon the increase in memory retention elicited by intra-hippocampal ghrelin (Carlini et al., 2007), meaning that low serotonin levels could be involved in the expression of ghrelin's effects. It has been suggested that serotonin and SSRI compounds inhibit LTP in various physiological assays (Corradetti et al. 1992; Mori et al. 2001; Kim et al. 2006). Carlini and coworkers propose that binding of ghrelin to its receptors in the hippocampus increases intracellular levels of calcium, stimulating NOS (nitric oxide synthase) activity and leading to an increase of nitric oxide (NO), which inhibits serotonin release (Ghersi et al. 2011). Another study recently showed that caloric restriction (known to increase ghrelin levels) seems to enhance fear extinction learning through a SERT (serotonin transporter)-dependent mechanism (Riddle et al. 2013).

Ghrelin was also recently shown to induce hippocampal neurogenesis in adult mice, which is known to be important in learning and memory (0.

1.5 Aims

Learning to locate food, remembering those locations and the strategy used to find them are evolutionarily important skills for survival (Moran & Gao 2006), especially in times of hunger when sources are scarce. This suggests that these abilities may be tied to signaling molecules involved in energy balance (Moran & Gao 2006), such as ghrelin. In fact, convincing links between ghrelin and memory have been found. Ghrelin was shown to increase memory retention (Carlini et al. 2004; Carlini et al. 2010; Chen et al. 2011b; Davis et al. 2010; Diano et al. 2006), promote formation of spines on hippocampal dendrites, increase LTP (0; Chen et al. 2011b) and promote the synaptic incorporation of GluA1 in hippocampal neurons (Ribeiro et al., unpublished data). Furthermore, GHS-R1a expression in the hippocampus seems to be regulated during development (Katayama et al. 2000), suggesting that it may play a role in hippocampal development.

The overall aim of this work is to unveil the mechanisms through which ghrelin can affect hippocampal-dependent learning and memory. In particular, we aimed to further clarify the role of GHS-R1a activity in hippocampal development, specifically on dendritic arborization and spine turnover and, in addition, we explored the effects of GHS-R1a activation on regulating hippocampal GluA1-AMPA receptors cell-surface levels.

2. MATERIALS AND METHODS

Animals and housing

Male C57BL6/J mice (8-10 weeks old) were used for the biotinylation assays and E18–E19 Wistar rat embryos were used for the primary cultures of rat hippocampal neurons.

Caloric restriction

Mice were singly-housed and maintained in a temperature-controlled environment on a 12-h light-dark cycle. Mice with 8-10 weeks of age were fed *ad libitum* for 2 days and the average daily food intake was determined. Mice were then divided into equal groups and were given either *ad lib* access to food (Control group) or to a wet pellet corresponding to 60% of the average daily food intake (Caloric Restriction group). All mice were weighed and fed daily at the same period of the day for a total period of 10 days.

Serum ghrelin levels determination

Mice were anesthesized with halothane and blood was collected from the decapitated animals. Serum was obtained by centrifugating the blood at $2000 \times g$ for 15min, at 4°C, and recovering the supernatant. Acylated ghrelin levels were determined from serum using ELISA kits as per the manufacturer's instructions (#B841522RA, Wuhan BIOABB).

Biotinylation assays

Hippocampal transverse slices (350 μm) obtained from C57BL/6 mice 8–10 weeks old were prepared using a tissue slicer. For biotinylation assays, 14-20 slices per mouse were used in case of the caloric restriction experiment, and 10 slices (5 per condition) were used for slices stimulation with MK-0677. The slices were equilibrated in ACSF (in mM: 124 NaCl, 3 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, 1.1 NaH_2PO_4, 2 $MgSO_4$, 10 D-Glucose, pH 7.4) for 15 min., on ice, in case of the caloric restriction experiment, or incubated for 2 h at 37°C in ACSF or 1 μM MK-0677 in ACSF, under continuous gasification with 5% CO_2/95% O_2, in case of slices stimulation with MK-0677.

Slices were transferred to ACSF solution containing 0.3 mg/ml NHS-SS-biotin (Pierce, Termo Fisher Scientific, Rockford, USA) and kept on ice with agitation for 45 min. Slices were then incubated in ACSF with 1 μM lysine to block all reactive NHS-SS-biotin in excess, and collected in 500 μl of lysis buffer [1% (v/v) Triton X-100, 0.1% (v/v) SDS, 1 mM EDTA, 50 mM NaCl, 20 mM Tris, pH 7.5, supplemented with 1 mM DTT, 0.1 mM PMSF, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin] and sonicated on ice for 30 s. Homogenates were centrifuged for 5 min at 4°C at 16,100 x g and supernatants collected. Protein concentration was quantified by the BCA method (Pierce, Termo Fisher Scientific, Rockford, USA), and the same amount of protein (500 μg) was used in all experimental conditions. To precipitate biotinylated proteins, 80 μl of Ultra-link immobilized neutravidin plus beads were added to samples for 2 h at 4°C, under constant agitation. Non-biotinylated proteins were removed by centrifugation at 2500 x g for 2 min, and beads were

washed three times with lysis buffer. Biotinylated proteins were eluted with denaturing buffer at 95°C for 5 min. Samples were processed for Western blotting analysis.

Protein extracts

Protein extracts were prepared in lysis buffer [10mM HEPES (pH 7.4), 150mM NaCl, 10mM EDTA, 1% (v/v) Triton X-100 supplemented with 1mM DTT, 0.1mM phenylmethylsulfonyl (PMSF), 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1μg/ml antipain, 1μg/ml pepstatin (CLAP) and a cocktail of phosphatase inhibitors (1x, Roche, Carnaxide, Portugal)]. After sonication and centrifugation at 16,100 × g for 10 min at 4°C, protein in the supernatant was quantified using the bicinchoninic acid (BCA) assay kit (Pierce, Termo Fisher Scientific, Rockford, IL, USA). The samples were denatured with 5x concentrated denaturating buffer [62.5 mMTris-HCl (pH 6.8), 10% (v/v) Glicerol, 2% (v/v) SDS, 0.01% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol (added fresh)] or with 2 x concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue), and boiled for 5 min.

Western Blotting

Protein extracts were resolved by SDS-PAGE in 7.5% polyacrylamide gels. For western blot analysis, proteins were transferred onto a PVDF membrane (Millipore, Madrid, Spain) by electroblotting (40 V, overnight at 4°C).

The membranes were blocked for 1 h at room temperature in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% (v/v) Tween-20 (TBS-T), and 5% (w/v) low-fat milk or BSA. Membranes were probed during 1 or 2 h at room temperature or overnight at 4°C, with the primary antibodies diluted in TBS-T containing 5% or 0.5% (w/v) low-fat milk or 5% (w/v) BSA. Following several washes, membranes were incubated for 45 min to 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 up to 5 min at room temperature. Membranes were scanned with the Storm 860 scanner (GE Heathcare, Carnaxide, Portugal), and quantified using the ImageQuant software under linear exposure conditions. When necessary, the membranes were stripped (0.2 M NaOH for 5 min) and re-probed.

Rat hippocampal neuron cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin [0.06% (w/v), 15 min, 37°C] (GIBCO, Invitrogen, Barcelone, Spain) in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution [5.36 mMKCl, 0.44 mM KH2PO4, 137 mM NaCl, 4.16 mM NaHCO3, 0.34 mM Na2HPO4·2H2O, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% (w/v) phenol red]. Hippocampal cells were washed with Hanks' balanced salt solution six times. The cells were mechanically dissociated and then plated in 6-well plates (8.9 x 10^4 cells/cm^2) coated with poly-D-lysine (0.1 mg/ml) for biochemical purposes or at a final density of 3 x 10^5 cells/dish on poly-D-lysine-coated coverslips in 60 mm culture dishes for imaging purposes. The cells were plated in neuronal plating medium [MinimumEssential Medium (MEM; GIBCO, Invitrogen,

Barcelone, Spain) supplemented with 10% (v/v) horse serum (GIBCO, Invitrogen, Barcelona, Spain), 0.6% (w/v) glucose, and 1 mM pyruvic acid]. Once neurons attached to the substrate, after 2–4 h, in case of high density cultures the neuronal plating medium was replaced by neuronal culture medium containing neurobasal medium (GIBCO, Invitrogen, Barcelona, Spain) supplemented with B27 supplement (1:50 dilution; GIBCO, Invitrogen, Barcelona, Spain), 25 μM glutamate, 0.5 mM glutamine, and 0.12 mg/ml gentamycin (GIBCO, Invitrogen, Barcelona, Spain). The coverslips were flipped over an astroglial feeder layer in 60 mm culture dishes containing neuronal culture medium. 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 overgrowth of glia, neuron cultures were treated with 5 μM cytosine arabinoside after 3 days. Cultures were maintained in a humidified incubator of 5% CO_2/95% air at 37°C, feeding the cells once per week by replacing one-third of the medium per well or dish, using neuronal culture medium without glutamate.

Transfection of cultured rat hippocampal neurons

Transfection of rat primary cultures of hippocampal neurons was carried out using the calcium phosphate transfection protocol (adapted from Jiang et al. 2004). The plasmids (2-4 μg per coverslip) were diluted in Tris-EDTA transfection buffer (10 mM Tris-HCl and 2.5 mM EDTA, pH 7.3). Briefly, a $CaCl_2$ solution (2.5 M in 10mM HEPES) was then added, dropwise, to the plasmid DNA-containing solution to give a final concentration of 250 mM $CaCl_2$. This was then added to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, 42 mM HEPES, pH 7). The mixture was vortexed gently for 2–3 s, and the precipitate was allowed to develop at room temperature for 30 min, protected from light, and vortexed every 5 min. The precipitated DNA was added dropwise to the coverslips, and the cultures were incubated with the precipitate for 3 h in the presence of kynurenic acid (2 mM). Each coverslip was transferred to a fresh well of the 12-well plate containing 1ml of conditioned culture medium with kynurenic acid (2 mM), slightly acidified with HCl (~5 mM final concentration), and the plate was returned to a 37°C, 5% CO 2/95% air incubator for 15–20 min. Coverslips were then transferred to the original dish containing the conditioned medium. The cells were then returned to a 37°C, 5% CO_2/95% air incubator to allow expression of the transfected construct. For cells receiving 3 day stimuli at 9DIV, transfection with GFP was performed at 5DIV, and cells transfected with hGHS-R1a (Human GFP-tagged GHS-R1a), GHS-R1a shRNA-GFP, luciferase shRNA-GFP, GHS-R1a shRNA-GFP and hGHS-R1a or mCherry and hGHS-R1a were transfected at 9DIV. In the case of the overexpression experiment, hGHS-R1a was GFP-tagged.
For plasmid-based RNA inhibition of GHS-R1a, the complementary oligonucleotides that target nucleotides 79–97 (gactcactgcctgacgaac) of rat GHS-R1a [(NM_032075); (adapted from Shrestha et al. 2009) were annealed and ligated into the HpaI/XhoI sites of the U6 promoter-driven short hairpin RNA expression vector pLentiLox3.7(CMV)EGFP that expresses EGFP under the CMV promoter. The luciferase shRNA-GFP targets firefly luciferase and was described previously [(Flavell et al., 2006); kind gift from Dr Michael E. Greenberg], *mCherry* fluorescent protein was cloned into the plentilox 3.7 vector (kind gift from Anne Marie's Craig lab, Vancouver), and the hGHS-R1a was cloned into the pcDNA3 vector [(Leung et al., 2007); kind gift from Helen Wise .

GHS-R1a stimulation

Hippocampal slices or neurons were treated with the GHS-R1a agonist MK-0677 (1µM), with MK-0677 (1µM) in the presence of the GHS-R1a antagonist [D-Lys3]-GHRP-6 (100 µM, added 30 min before the agonist), with [D-Lys3]-GHRP-6 (100 µM) alone or with the GHSR1a inverse agonist [(D-Arg1,D-Phe5,D-Trp7,9,Leu11)-substance P] (1 µM). Hippocampal neurons in culture were incubated for 1 at 18-19 DIV or 20h at 19 -20DIV or for 3 days at 9DIV. Hippocampal slices were incubated for 2h.

Immunocytochemistry

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 4°C. Neurons were then incubated in 10% (w/v) BSA in PBS for 30 min at 37°C to block nonspecific staining, and incubated in appropriate primary antibodies diluted in 3% (w/v) BSA in PBS (2 h, 37°C or overnight, 4°C). After washing 6 times in PBS, cells were incubated with the secondary antibodies diluted in 3% (w/v) BSA in PBS (1 h, 37°C). After 6 more washes in PBS, the coverslips were mounted using fluorescent mounting medium from DAKO (Glostrup, Denmark).

Microscopy and image analysis

Images were acquired on a widefield Axio Observer and on a LSM710 confocal microscope (Carl Zeiss, Gemany), using either a 20× Plan-ApoChromat objective (N.A. 0.8) or a 63× Plan-ApoChromat oil objective (N.A. 1.4). Images were quantified using image analysis software Fiji and Neurolucida.

To ensure that the data analyses were performed by an experimenter unaware of the treatment condition, each sample was coded by an independent observer, and the code was not broken until all analyses were completed.

For quantitation, sets of cells were cultured and stained simultaneously, and seven to ten cells per condition were imaged using identical settings, for each independent experiment.

The protein signals were analyzed after thresholds were set, such that recognizable clusters were included in the analysis. Regions around thresholded puncta were overlaid as a mask in the Vglut1 channel, and colocalization was determined. For quantifying the protein signals in transfected neurons, fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons. Measurements were performed in three or four independent preparations.

For analysis of F-actin organization, after labeling hippocampal neurons with Actin-stain 555 phalloidin, F-actin clusters were defined operationally as 0.14-1.42 μm^2 F-actin-enriched puncta along dendrites (with an average pixel intensity at least 50% above that in the adjacent dendritic region).

Sholl analysis and spine morphology were performed with Neurolucida software. For the Sholl analysis images were acquired with the 20× objective with the widefield Axio Observer in order to sample the entire neuron. With Neurolucida, each neuron was traced and then the spatial distribution and length of dendritic branches were analyzed using the concentric analysis of Sholl,

performed using the Neuroexplorer component of the Neurolucida program. The data were reanalyzed by counting the number of occurrences of branch points in the dendritic arbor falling between concentric spheres separated by a fixed number of microns (10 μm).

For spine morphology, dendritic segments (67.5 μm in length), were imaged on the confocal microscope using the 63× oil objective. All confocal stacks were acquired at 1024 × 1024 pixel resolution with a z-step of 0.3 μm, a pinhole setting of one Airy unit and optimal settings for gain and offset. On average 1-2 z-stacks were imaged per neuron, with a total of 7-10 neurons imaged per condition in each independent experiment.

On the basis of morphology, spines were classified into the following categories: i) Thin: spines with a long neck and a visible small head; ii) Mushroom: spines with a well-defined neck and a more voluminous head; iii) Stubby: spines without a distinguishable neck, with stubby appearance; and iv) Branched: spines with two or more protrusions; and (v) Filopodia: long neck and a very small or absent head, non-colocalized with PSD95.

Statistics

Mann-Whitney tests were used to compare statistical differences between any two groups. Comparisons between multiple groups were performed with the Kruskal-Wallis analysis of variance followed by Dunn's Multiple Comparison. In Sholl analysis, data were analyzed per circumference.

3. RESULTS

3.1. GHS-R1a activity affects hippocampal neuron dendrites and spines during development

3.1.1. GHS-R1a expression increases during development

GHS-R1a expression pattern seems to be regulated during development in the hippocampus (Katayama et al. 2000), suggesting that it may play a role in hippocampal development. In order to characterize the expression pattern of this receptor in our system, protein extracts from hippocampal neurons in culture at 7, 15, 19 and 21 DIV were analyzed by western blot. During the analyzed period of time we observed a consistent increase in GHS-R1a levels (fig. 1).
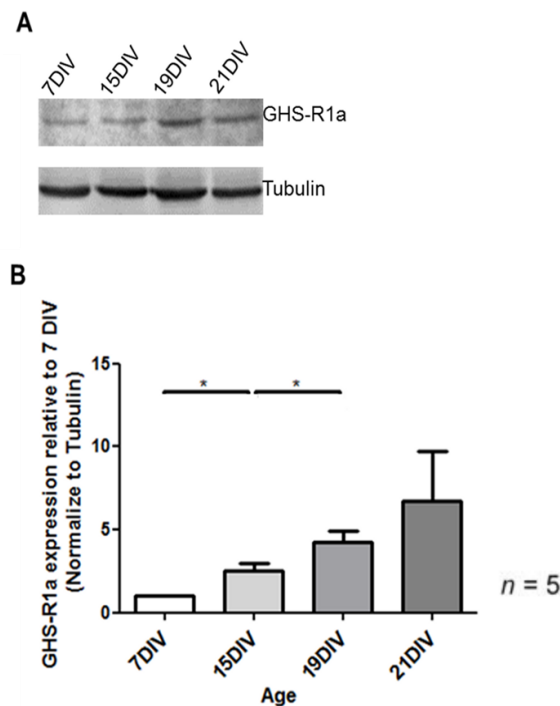
Figure 1: Developmental profile for the expression of GHS-R1a in hippocampal cultured neurons. A: Representative western blots for GHS-R1a and tubulin (loading control). **B:** Mean intensity of GHS-R1a (normalized to tubulin and expressed in % of control). There was a significant increase in GHS-R1a expression from 7 to 15 and from 15 to 19 DIV, as assessed by Paired t test. Error bars represent s.e.m and *n* represents the number of independent experiments.

3.1.2. GHS-R1a activity plays a role in dendritic arborization

GHS-R1a activation has been shown to enhance LTP and spine formation in hippocampus (Chen et al. 2011b; Diano et al. 2006) and some of the molecules involved in these processes, such as Ras, ERK, PI3K, Akt, Sept7, BDNF, PKA, CREB, CaMKII and CaMKIV, are also involved in dendritic arborization (Qin et al. 2005; reviewed in Urbanska et al. 2008; Jaworski et al. 2005; Brand et al. 2012b; Tyler & Pozzo-Miller 2003; Caldeira et al. 2007; Sato et al. 2011; reviewed in Santos et al. 2008; Minichiello 2009; Wayman et al. 2006; Redmond et al. 2002; Fink et al. 2003). Furthermore, some of GHS-R1a's downstream signaling pathways (partially) overlap with some of the pathways

that have been shown to be involved in dendritic arborization. Therefore, and because GHS-R1a's expression increases during development, we sought to investigate whether GHS-R1a plays a role in dendritic arborization.

Constitutive activity

Having into account that GHS-R1a has a high constitutive activity, and that sometimes this type of activity parallels the agonist-induced activity, we investigated the effects of GHS-R1a constitutive activity on dendritic arborization. We chose to transfect hippocampal neurons at 9 DIV since it is a time point in which the branching of neurons is still highly dynamic (Chen et al. 2011a), allowing us to successfully interfere with the arborization mechanism and, on the other hand, as seen in figure 1, GHS-R1a is already expressed at a satisfactory amount. Neurons were transfected with GHS-R1a shRNA-GFP to induce a knockdown of the receptor, whereas control neurons were transfected with luciferase shRNA-GFP to rule out any side effects induced by activation of the shRNA machinery. The GHS-R1a shRNA plasmid promotes an effective knockdown of the receptor, to about 70% of the endogenous expression level (fig. 1S), although it should be mentioned that in this case, expression occurred during 4 days more than in the present experiment. After 3 days of expression, at 12 DIV, neurons were immunolabeled for GFP and Sholl analysis was performed. We observed a significant decrease in arborization complexity in the neurons where GHS-R1a was knocked down, in the regions closest to the soma (20-80μm distance from the cell body) (fig. 2A,B), as assessed by the number of neurite intersections. In order to exclude off-targets effects of the GHS-R1a shRNA, we co-transfected neurons with GHS-R1a shRNA-GFP and human GHS-R1a-GFP [(hGHSR-GFP); the human form of GHS-R1a is not a target for the shRNA used and, as shown in figure 3, it is efficiently expressed by neurons]. As shown in figure 2A and B, in this condition the neurite intersection number is not significantly different from control levels (at 40-80μm from the soma), indicating that the decrease in the arborization complexity was mediated by the ghrelin receptor. The GHS-R1a knockdown also led to a decrease in total dendritic length, although this was not totally prevented by co-expression of the rescue hGHSR-GFP construct (fig. 2C). However, there certainly seems to be a tendency towards rescue, especially considering that the rescue condition was not different from the control.

These results strongly suggest that the constitutive activity of the GHS-R1a is important for dendritic arborization, since it is unlikely that there is ghrelin in the culture medium that could activate the ghrelin receptor.

Since abolishing GHS-R1a expression or function decreases dendritic arborization complexity, we sougth to investigate whether overexpression of the receptor would have the opposite effect. We transfected hippocampal neurons at 9 DIV with hGHS-R1a-GFP and mCherry, and control neurons, were transfected with luciferase shRNA-GFP (as in the knockdown experiment). After 3 days of expression, at 12 DIV, neurons were immunolabeled for GFP, and Sholl analysis was performed. Surprisingly, GHS-R1a overexpression did not cause an increase in dendritic arborization (fig. 3). One possible reason for this unexpected result is a potential saturation, at this stage of development, of the downstream signaling pathways of GHS-R1a mediating the effects on dendritic arborization.
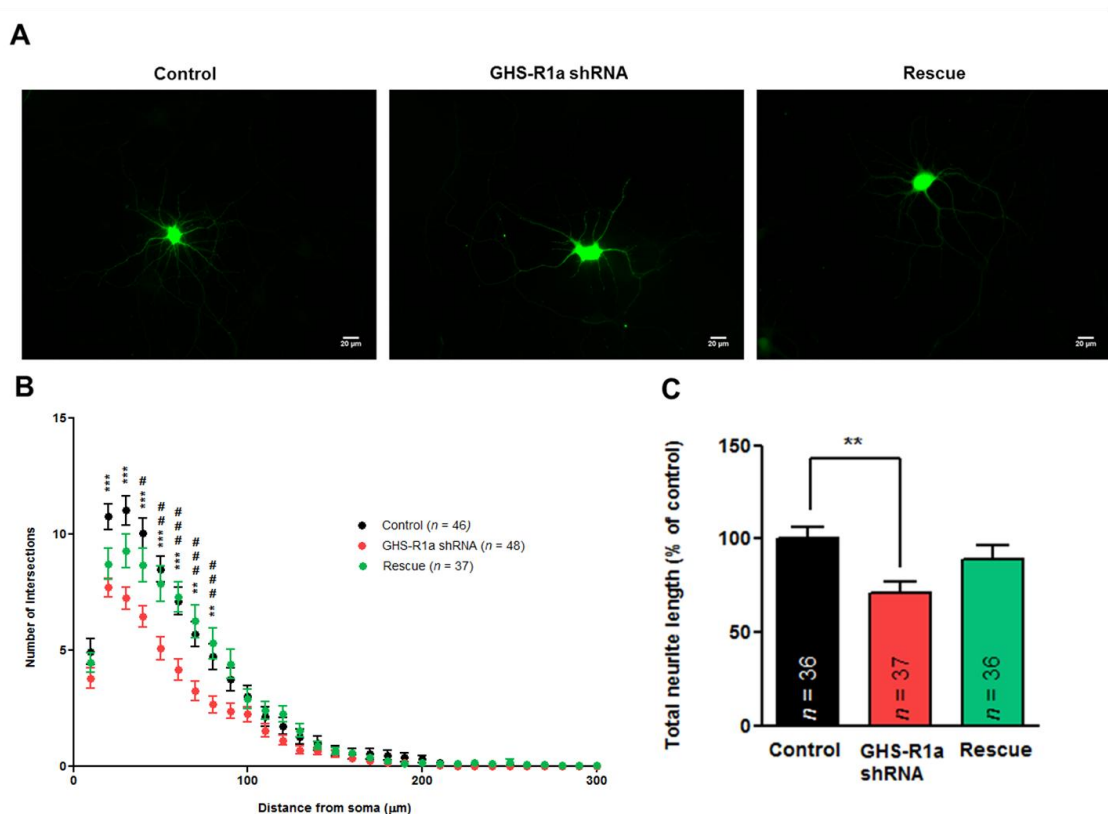
Figure 2: Effects of GHS-R1a knockdown on dendritic arborization in hippocampal neurons. A: Representative immunofluorescence images of neurons transfected at 9 DIV with (from left to right) Luciferase shRNA-GFP (control), GHS-R1a shRNA-GFP (knockdown) or GHS-R1a shRNA-GFP + hGHS-R1a-GFP (rescue). At 12 DIV neurons were stained for GFP. **B:** The number of neurite intersections at different distances from the soma as determined by Sholl analysis. The 3 conditions were analyzed for statistical significance per circumference (different distances from the soma) using Kruskal-Wallis tests (20µm: ***p = 0.0002; 30-70µm: ***p < 0.0001; 80µm: ***p = 0.0003). There was a significantly lower number of intersections at 20–80 µm distance from the soma in GHS-R1a knockdown neurons (20-60µm: ***p < 0.001; 70-80µm: **p < 0.01) compared to control neurons. The rescue condition increased the number of intersection relative to the knockdown condition in intersections 40-80µm (40µm: #p < 0.05; 50µm: ##p < 0.01; 60-80µm: ###p < 0.001). The rescue is no different from the control except at 40µm (*p < 0.05), where there is only a partial rescue. Results are averaged from 4 independent experiments. **C:** The total neurite length as determined by Sholl analysis. Results are expressed as % of control and averaged from 3 independent experiments. Statistical significance was determined with a Mann Whitney test. GHS-R1a knockdown neurons have a lower total dendrite length as compared to the control (**p < 0.01). There are no statistical differences between the rescue and the control nor between the rescue and the knockdown. **(B,C):** Error bars represent s.e.m and *n* represents the total number of analysed cells.

3.1.3. GHS-R1a activity plays a role in filopodia formation

Having determined that GHS-R1a activity seems to play a role in dendritic arborization, and considering that some of the molecules involved in arborization are also involved in spine formation and LTP, two processes shown to be enhanced by GHS-R1a activity, we decided to assess the effects of ghrelin and its receptor on spine number and morphology. This seems especially interesting considering that LTP is often paired with structural alterations in spines, thought to occur mostly in the late phase of LTP, and that ghrelin was shown to specifically enhance the maintenance of late-phase of LTP in the DG (Chen et al. 2011b) (dependent on protein synthesis). Our own data show that ghrelin receptor activation enhances LTP in the CA3-CA1 synapse and the effect is higher for the later phases of LTP (>20 min after LTP induction) (Luís Ribeiro, personal communication).
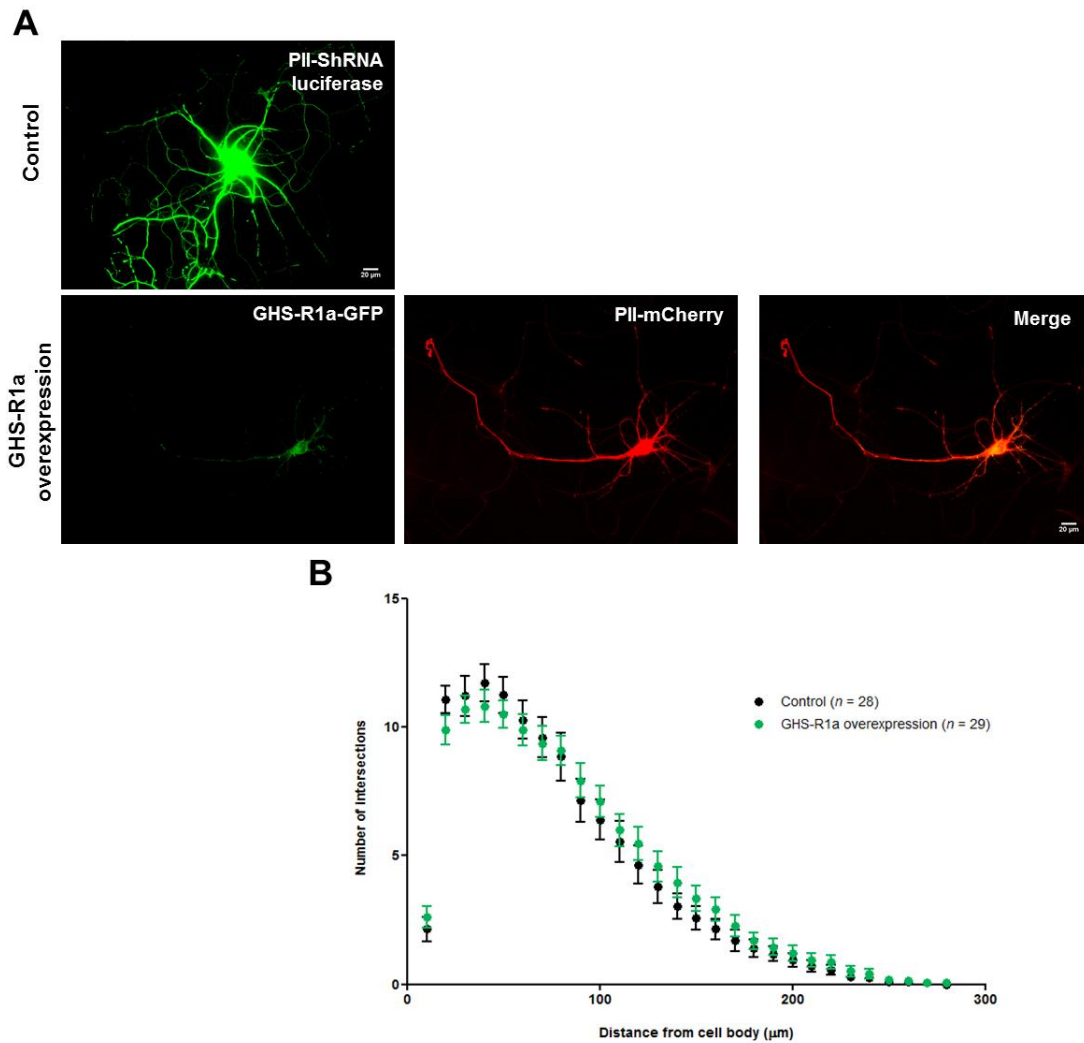
Figure 3: Effects of GHS-R1a overexpression on dendritic arborization in hippocampal neurons. **A:** Representative immunofluorescence images of neurons transfected at 9 DIV with (from top to bottom) Luciferase shRNA-GFP (control) or mCherry and hGHS-R1a-GFP (overexpression). At 12 DIV neurons were stained for GFP. **B:** The number of neurite intersections at different distances from the soma as determined by Sholl analysis. The 2 conditions were analyzed for statistical significance per circumference (different distances from the soma) using Mann Whitney tests. There were no significant differences. Results are averaged from 3 independent experiments. Error bars represent s.e.m and *n* represents the total number of analysed cells.

Constitutive activity

We first looked at the effect of the receptor's constitutive activity at the level of spine morphology. Again, hippocampal neurons were transfected at 9 DIV with GHS-R1a shRNA-GFP to induce a knockdown of the receptor, and control neurons were transfected with luciferase shRNA-GFP to rule out any effects induced by activation of the shRNA machinery. For the rescue condition neurons were co-transfected with GHS-R1a shRNA-GFP and hGHS-R1a. After 3 days of expression, at 12 DIV, neurons were immunolabeled for GFP and PSD-95. Z-stack images were acquired and Neurolucida software was used to classify spines into 5 distinct categories: i) Thin: spines with a long neck and a visible small head; ii) Mushroom: spines with a well-defined neck and a more voluminous head; iii) Stubby: spines without a distinguishable neck, with stubby appearance; and iv) Branched: spines with two or more protrusions; and (v) Filopodia: long neck and a very small or absent head,

non-colocalized with PSD95 (fig. 4). We observed no effect of the GHS-R1a knockdown on the number of PSD-95 colocalized spines (fig. 5B), but the number of filopodia significantly decreased in the knockdown, an effect rescued by co-expression of the GHS-R1a resistant to shRNA (fig. 5C). We also analyzed the proportions of each type of spine relatively to total spine number, and observed that the knockdown caused a decrease in the proportion of filopodia, and an increase in the proportion of mushroom and stubby spines (traditionally classified as more mature spines) (fig. 5D), suggesting a role for the ghrelin receptor in spine/synapse formation or turnover. In the rescue conditions there were no significant differences from the control condition, indicating a specific effect of the GHS-R1a knockdown. Considering that the total number of PSD-95-colocalized spines did not increase, the observed higher proportion of mushroom and stubby spines could be only a relative effect due to the decrease of the total number of filopodia.

Treatment of neurons (transfected with GFP at 5 DIV) with the GHS-R1a inverse agonist substance P (1µM) from 9 to 12 DIV did not cause an effect on the number of PSD-95-containing spines (fig. 6B) or on the number of filopodia (fig. 6C). It did, however, increase the proportion of mushroom spines (fig. 6D).

Altogether, these data indicate that the expression of the GHS-R1a in hippocampal neurons is playing a role in promoting the formation of filopodia, since knock-down of the receptor decreased the number of dendritic filopodia. This effect could be due to the constitutive activity of the ghrelin receptor, but treatment with the inverse agonist failed to reproduce this effect. One possibility is that the role of the ghrelin receptor in promoting dendritic filopodia formation may depend on its heteromerization with other receptors, and therefore on triggering signaling pathways that are not blocked by the receptor inverse agonist. Nevertheless, these data are at the moment still preliminary and more experiments should be performed.
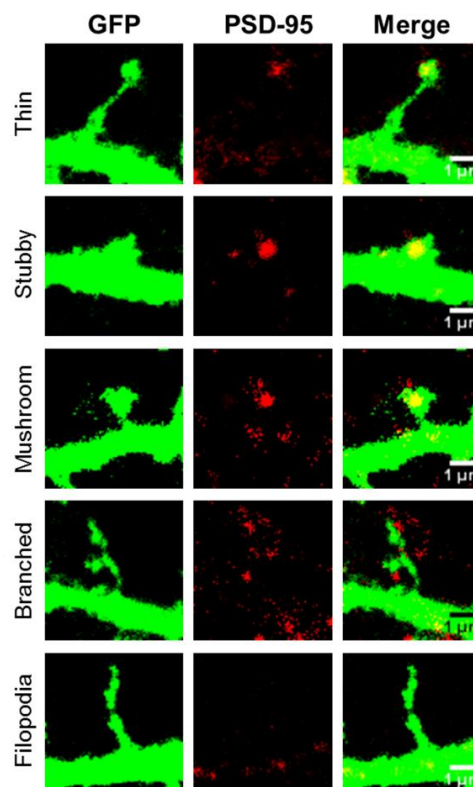
Figure 4: Typical examples of each type of spine. Thin, stubby, mushroom and branched spines colocalize with PSD-95, whereas filopodia do not.
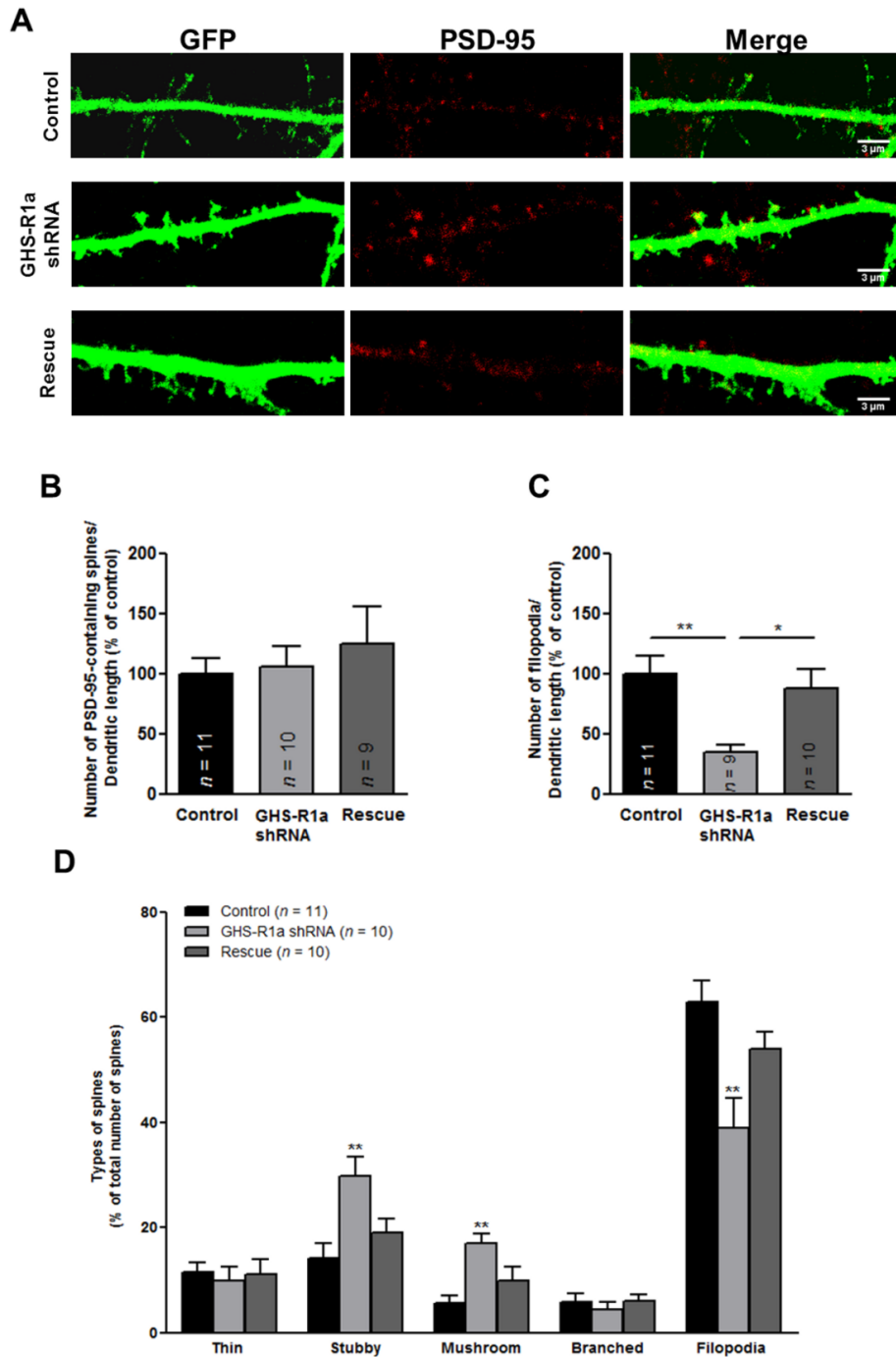
Figure 5: Effects of GHS-R1a knockdown on number and types of spines in hippocampal neurons. **A:** Representative immunofluorescence images of neurons transfected at 9 DIV with (from top to bottom) Luciferase shRNA-GFP (control), GHS-R1a shRNA-GFP (knockdown) or GHS-R1a shRNA-GFP + hGHS-R1a (rescue). At 12 DIV neurons were stained for GFP and PSD-95 and number and type of spines were quantified using Neurolucida software. **B:** The number of PSD-95-containing spines was no different between conditions. Results are expressed as % of control. **C:** There were significant differences in the number of filopodia (**$p = 0.0075$): GHS-R1a knockdown neurons had a smaller number of filopodia (**$p < 0.01$), an effect efficiently rescued by the hGHS-R1a (*$p < 0.05$). Results are expressed as % of control. D: The proportions (in % of total number of spines) of different types of spines varied (stubby: *$p = 0.,0104$; mushroom: **$p = 0.,0017$; filopodia: *$p = 0.,0110$). The GHS-R1a knockdown neurons had a larger proportion of stubby and mushroom spines, and a smaller proportion of filopodia relative to control (**$p < 0.01$). The rescue condition did not significantly differ from either of the other conditions. **B, C, D:** All sets of data were analyzed by a Kruskal-Wallis test. Error bars represent s.e.m and *n* represents the total number of analyzed cells. Results are averaged from 1 independent experiment.

Agonist-induced activity

We then looked at the effects of agonist-induced GHS-R1a activity on spine morphology. We treated neurons (transfected with GFP at 5 DIV) with the non-peptide GHS-R1a agonist MK-0677 (1μM) or with MK-0677 in the presence of the receptor antagonist [D-Lys3]-GHRP-6 (100μM), from 9 to 12 DIV. Interestingly, treatment with the agonist induced the opposite effect as the knockdown of the receptor. We saw no effects on PSD-95 containing spines (fig. 7B) but a significant increase in the number of filopodia (fig. 7C). The proportion of filopodia increased (an effect blocked by the antagonist, indicating it is mediated by GHS-R1a), while the proportion of stubby spines decreased (fig. 7D). Considering that the total number of PSD-95-colocalized spines didn't decrease, the observed lower proportion of stubby spines could be only a relative effect due to the decrease of the total number of filopodia. It will be important to repeat these experiments in several more independent preparations.

Altogether, these results suggest that both the expression of the ghrelin receptor and its agonist-induced activity seem to be important for new spine/synapse formation or turnover.
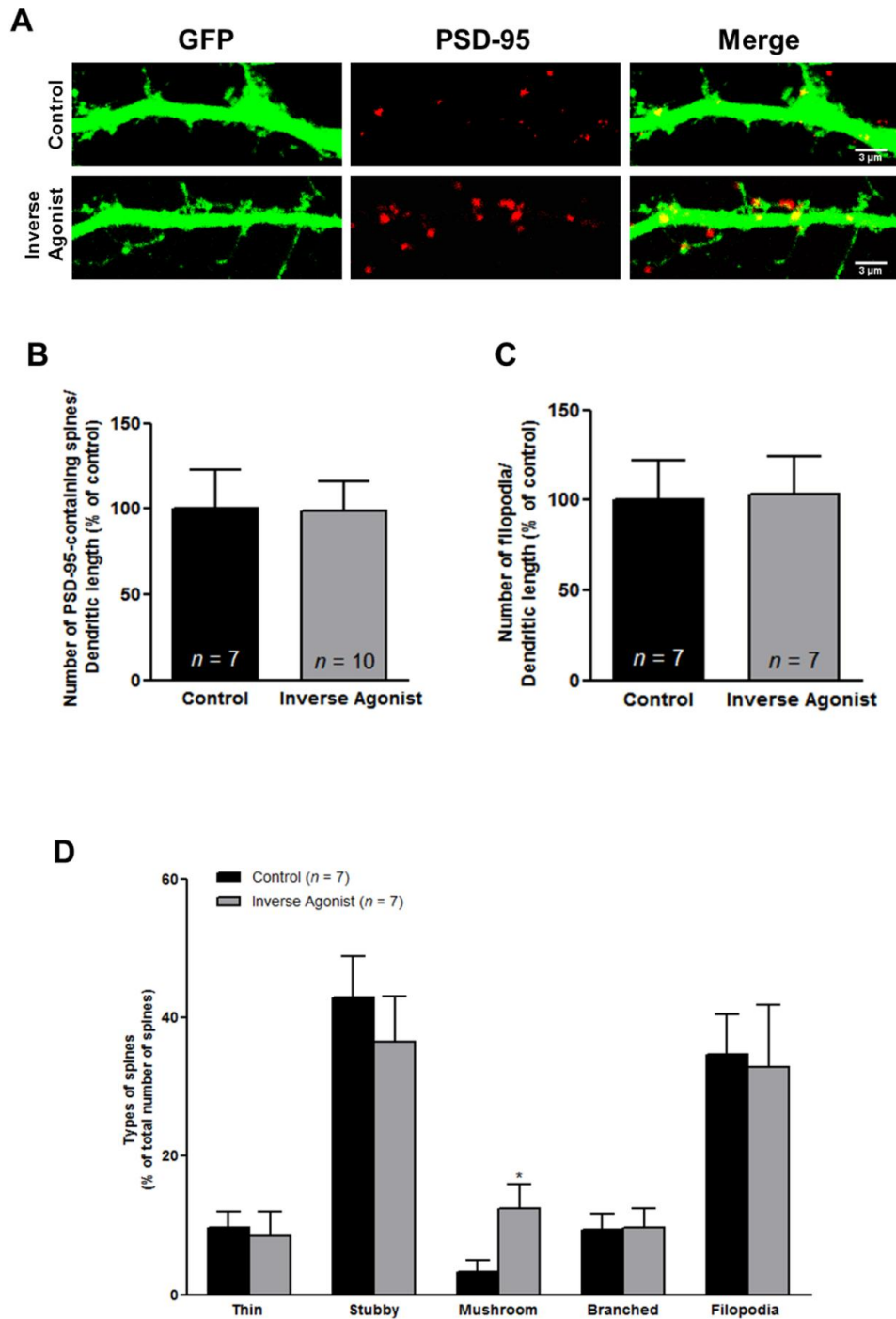
Figure 6: Effects of GHS-R1a inverse agonist on the number and types of spines in hippocampal neurons.
A: Representative immunofluorescence images of control (top) or inverse agonist-treated (bottom) neurons, transfected at 5 DIV with GFP. Stimuli were given at 9 DIV and at 12 DIV neurons were stained for GFP and PSD-95. Number and type of spines were quantified using Neurolucida software. **B:** The number of PSD-95-containing spines was no different between conditions. Results are expressed as % of control. **C:** The number of filopodia was also constant. Results are expressed as % of control. **D:** The proportion (in % of total number of spines) of mushroom spines increased in inverse agonist-treated neurons (*p = 0,0426). **B, C, D:** Data were analyzed by Mann-Whitney tests. Error bars represent s.e.m and *n* represents the total number of analyzed cells. Results are averaged from 1 independent experiment.

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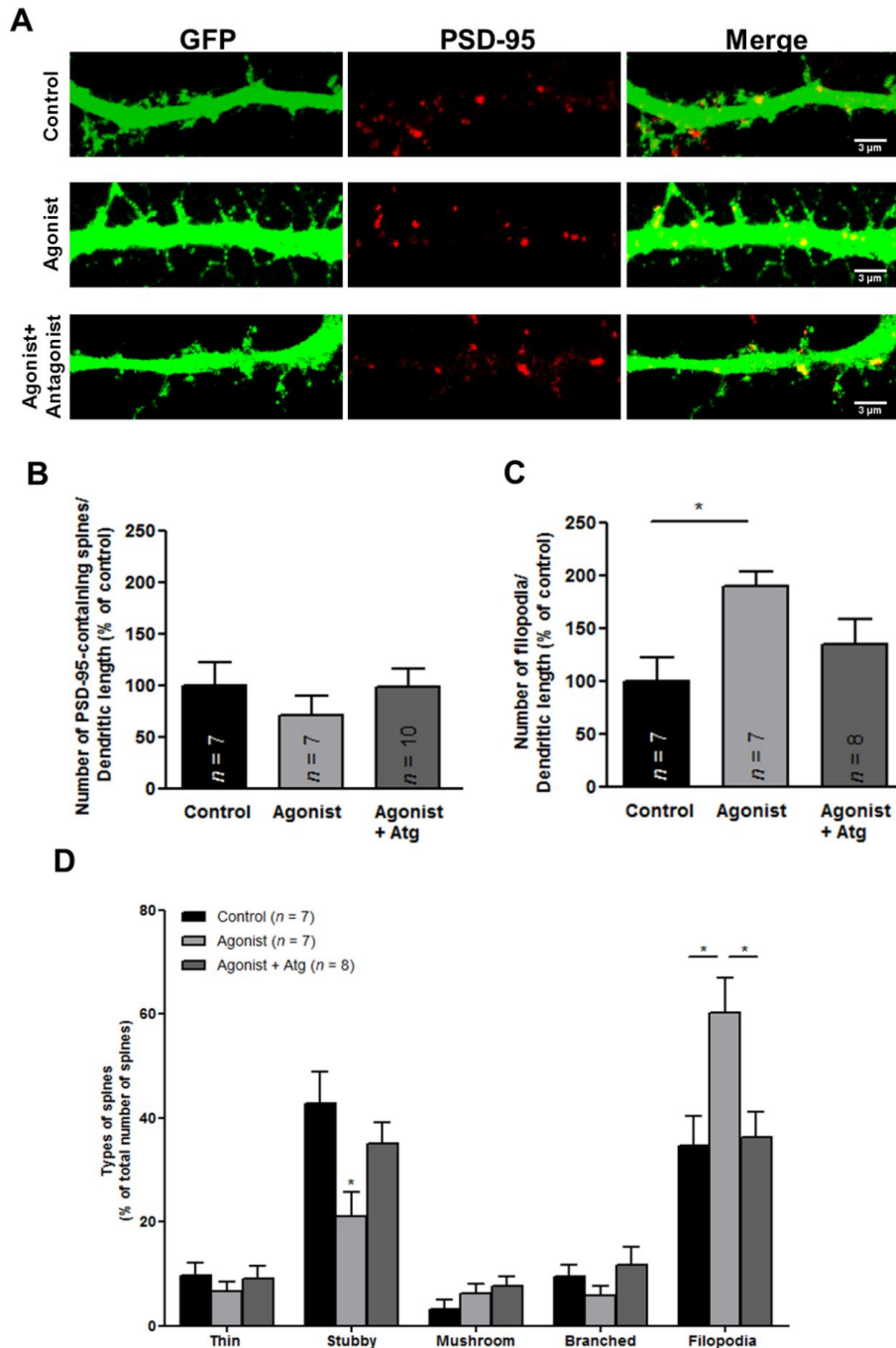
Figure 7: Effects of the GHS-R1a agonist on the number and types of spines in hippocampal neurons. A: Representative immunofluorescence images of (from top to bottom) control, agonist-treated, or agonist + antagonist-treated neurons, transfected at 5 DIV with GFP. Stimuli were given at 9 DIV and at 12 DIV neurons were stained for GFP and PSD-95. Number and type of spines were quantified using Neurolucida software. **B:** The number of PSD-95-containing spines was not different between conditions. Results are expressed as % of control. **C:** The number of filopodia differed significantly between conditions (*p = 0,0364). The agonist caused an increase in the number of filopodia relative to control (*p < 0.05) The rescue condition was not significantly different from control. Results are expressed as % of control. **D:** The proportions (in % of total number of spines) of different types of spines varied (stubby: *p = 0,0403; filopodia: *p = 0,0163). The agonist-treated neurons had a smaller proportion of stubby spines, and a larger proportion of filopodia relative to control (*p < 0.05). The agonist+antagonist condition did not significantly differ from control in either condition; in case of filopodia the antagonist significantly blocked the agonist-induced increase in number (*p < 0.05). **B, C, D:** All sets of data were analyzed by a Kruskal-Wallis test. Error bars represent s.e.m and n represents the total number of analyzed cells. Results are averaged from 1 independent experiment.

3.1.4. GHS-R1a activity plays a role in the accumulation of synaptic proteins

As GHS-R1a activity seems to have an effect on spines, we sought to investigate whether this effect would correlate with the accumulation of synaptic proteins.

Constitutive activity

We looked at the levels of Vglut1, a presynaptic vesicular transporter for glutamate, and of PSD-95, a postsynaptic scaffold protein, in neurons transfected at 9 DIV with GHS-R1a shRNA-GFP (knockdown), luciferase shRNA-GFP (control), or GHS-R1a shRNA-GFP and hGHSR-GFP (rescue). Neurons were immunolabeled at 12 DIV for GFP, PSD-95 and Vglut1. We observed a significant decrease in the total number of PSD-95 (fig 8) and Vglut particles (fig 8), which may mean that these proteins either accumulate less at the synapse when GHS-R1a levels are reduced, or are less produced. The rescue effectively blocked the effects of the knockdown (fig. 8), indicating that they are mediated by the ghrelin receptor. Considering that GHS-R1a activity also causes an increase in new filopodia, this could mean that the receptor is important for the formation of new filopodia and, later, their development into mature spines, through synaptic protein accumulation.

Treatment of neurons (transfected with GFP at 5 DIV) with the GHS-R1a receptor inverse agonist substance P (1µM) from 9 to 12 DIV did not cause a significant effect on PSD-95 (fig. 9B) or Vglut1 (fig. 9C) cluster number. However, there does seem to be a tendency for a decrease on the clustering of these synaptic proteins when the constitutive activity of GHS-R1a is decreased, which may become significant with an increased number of independent experiments.
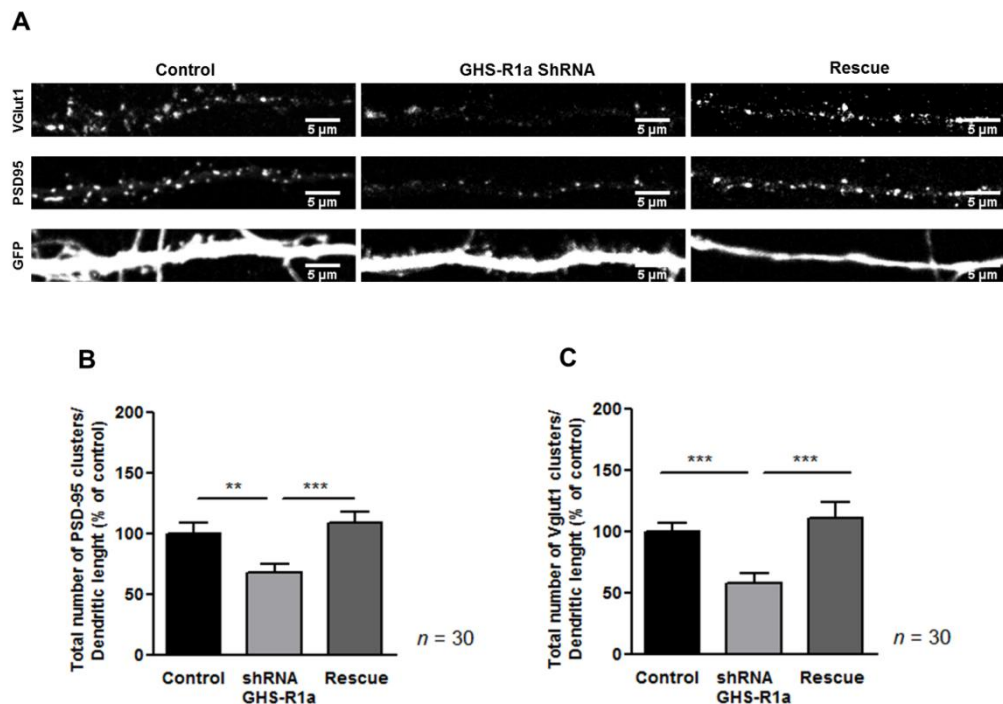
Figure 8: Effects of GHS-R1a knockdown on synaptic markers in hippocampal neurons. A: Representative immunofluorescence images of neurons transfected at 9 DIV with Luciferase shRNA-GFP (control), GHS-R1a shRNA-GFP (knockdown) or GHS-R1a shRNA-GFP + hGHS-R1a (rescue). At 12 DIV neurons were stained for PSD-95 and Vglut1. **B:** The number of PSD-95 clusters significantly differed between conditions (***p = 0,0006). The number of PSD-95 clusters significantly decreased compared to control (**p < 0.01) and this effect was efficiently rescued (***p < 0.001). **C:** The number of Vglut1 clusters significantly differed between conditions (***p < 0.0001). The number of Vglut1 clusters significantly decreased compared to control (***p < 0.001) and this effect was efficiently rescued (*** p < 0.001). **B, C:** Data were analyzed by a Kruskal-Wallis test. Error bars

represent s.e.m and *n* represents the total number of analyzed cells. Results are expressed as % of control and averaged from 3 independent experiments.
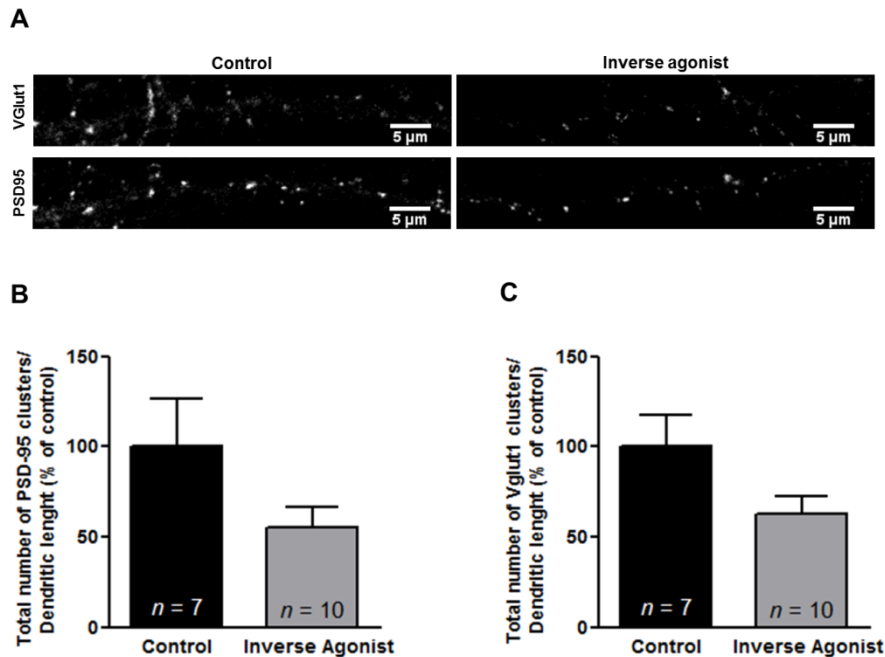
Figure 9: Effects of GHS-R1a inverse agonist on synaptic proteins in hippocampal neurons. A: Representative immunofluorescence images of control neurons and neurons treated with the inverse agonist at 9 DIV. At 12 DIV neurons were stained for PSD-95 and Vglut1. **B:** There were no significant differences in the number of PSD-95 clusters. **C:** The number of Vglut1 clusters also did not differ significantly between conditions **B, C:** Data were analyzed by a Mann-Whitney test. Error bars represent s.e.m and *n* represents the total number of analyzed cells. Results are expressed as % of control and averaged from 1 independent experiment.

3.2. GHS-R1a activity increases synaptic markers in mature hippocampal neurons

As GHS-R1a is even more highly expressed in older neurons, we further sought to determine whether GHS-R1a affects spines specifically during development, or whether it plays a similar role in mature neurons.

Constitutive activity

19-20 DIV hippocampal neurons were stimulated for 20 h with the GHS-R1a inverse agonist substance P (1µM), and neurons were immunolabeled for Vglut1, PSD-95 and F-actin (the cytoskeletal component of spines) was labeled with Actin-stain 555 phalloidin. We observed no differences in the F-actin cluster (fig. 10A) number nor total area (fig. 10B), but a significant decrease in PSD-95 (fig. 10C) and Vglut1 (fig. 10D) fluorescence intensity. This may indicate that GHS-R1a constitutive activity is involved in spine maturation in older neurons.
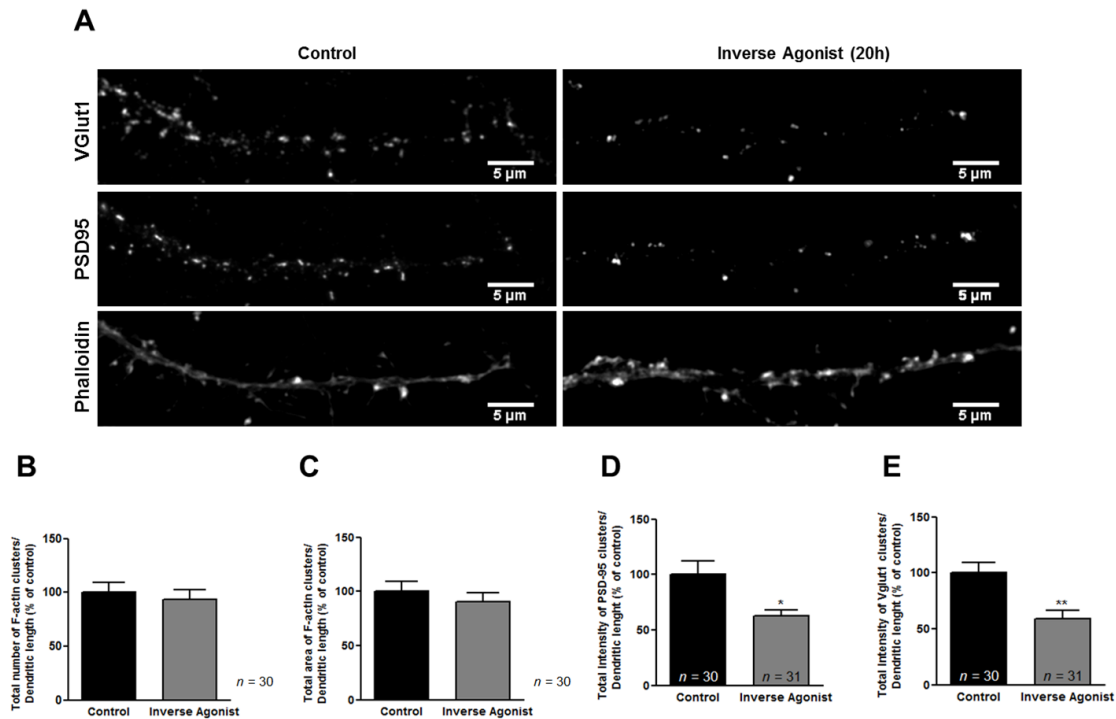
Figure 10: Effects of GHS-R1a inverse agonist on F-actin clusters and synaptic proteins in mature hippocampal neurons. A: Representative immunofluorescence images of control (left) and GHS-R1a inverse agonist-treated, 19-20 DIV neurons. 20 h stimuli were given at 18-19 DIV and at 19-20 DIV neurons were stained for Vglut1 (top), PSD-95 (middle) and F-actin (with Actin-stain 555 phalloidin, bottom). **B:** The number of F-actin clusters was not different between conditions. **C:** The total area of of F-actin clusters was also constant. **D:** The inverse agonist caused a decrease in PSD-95 total fluorescence intensity (*p < 0.05). **E:** Similarly, it caused a decrease in Vglut1 total fluorescence intensity (**p < 0.01). **B, C, D, E:** All sets of data were analyzed by Kruskal-Wallis tests. Error bars represent s.e.m and *n* represents the total number of analyzed cells. Results are expressed as % of control and averaged from 3 independent experiments.

Agonist-induced activity

19-20 DIV hippocampal neurons were stimulated for 1 or 20 h with the GHS-R1a agonist MK-0677 (1µM) or with MK-0677 in the presence of the GHS-R1a antagonist [D-Lys3]-GHRP-6 (100µM). 1 h stimulation with the agonist caused no significant effect on the total number of F-actin clusters (fig. 11B), but caused an increase in their area (fig. 11C). There was also an increase in PSD-95 puncta fluorescence intensity, an effect blocked by the GHS-R1a antagonist (fig. 12B), whereas Vglut puncta fluorescence intensity did not alter significantly (fig. 12C).

Figure 11: Effects of GHS-R1a activation on F-actin clusters in mature hippocampal neurons. A: Representative immunofluorescence images of control (left) and agonist-treated (right), 19-20 DIV neurons. 1 h stimuli were given at 19-20 DIV and neurons were stained for F-actin (with Actin-stain 555 phalloidin). **B:** The number of F-actin clusters wasn't significantly different between conditions. **C:** The total area of of F-actin clusters increased in agonist treated neurons relative to control (*p = 0,0445). **B, C:** Data were analyzed by a Mann-Whitney test. Error bars represent s.e.m and *n* represents the total number of analyzed cells. Results are expressed as % of control and averaged from 2 independent experiments.

After 20 h of stimulation with MK-0677, we observed an increase in the number of F-actin clusters (fig. 13B), but a significant effect on their area, which was observed after 1h stimulation (fig. 11C), was not observed (fig. 13C). The effect on F-actin cluster number was partially blocked by the GHS-R1a antagonist. The number of PSD-95 clusters increased after 20h activation of the GHS-R1a receptor, an effect blocked by the receptor antagonist (fig. 13D). The number of Vglut clusters was also significantly increased by activation of the GHS-R1a for 20h, but this effect was not blocked by the receptor antagonist(fig. 13E). These results suggest an increase in spine number after prolonged stimulation of the ghrelin receptor. Sustained elevated levels of ghrelin occur after caloric restriction (Cummings et al. 2002) and in conditions of chronic stress (Lutter et al., 2008), and may lead to effects such as those observed after 20h with the ghrelin receptor agonist.

Taken together, these results suggest that both GHS-R1a constitutive and agonist-induced activity have a role on promoting spine maturation, an effect consistent with the idea that the GHS-R1a activity plays a role in synaptic plasticity.

Figure 12: Effects of GHS-R1a activation on PSD-95 and Vglut1 in mature hippocampal neurons. A: Representative immunofluorescence images of (from left to right) control, agonist-treated, agonist + antagonist-treated, or antagonist-treated 19-20 DIV neurons. 1 h stimuli were applied and neurons were stained for Vglut1 (top) and PSD-95 (bottom). **B:** PSD-95 fluorescence intensity was significantly different between conditions (***$p < 0.0001$). The agonist caused an increase in PSD-95 fluorescence intensity (*$p < 0.05$), an effect blocked by co-application of the antagonist ($p < 0.05$). Furthermore, the antagonist alone did not differ from control condition. **C:** In Vglut total fluorescence intensity, none of the conditions differed from the control. **B, C:** Data were analyzed by Kruskal-Wallis tests. Error bars represent s.e.m and *n* represents the total number of analyzed cells. Results are expressed as % of control and averaged from 3 independent experiments.

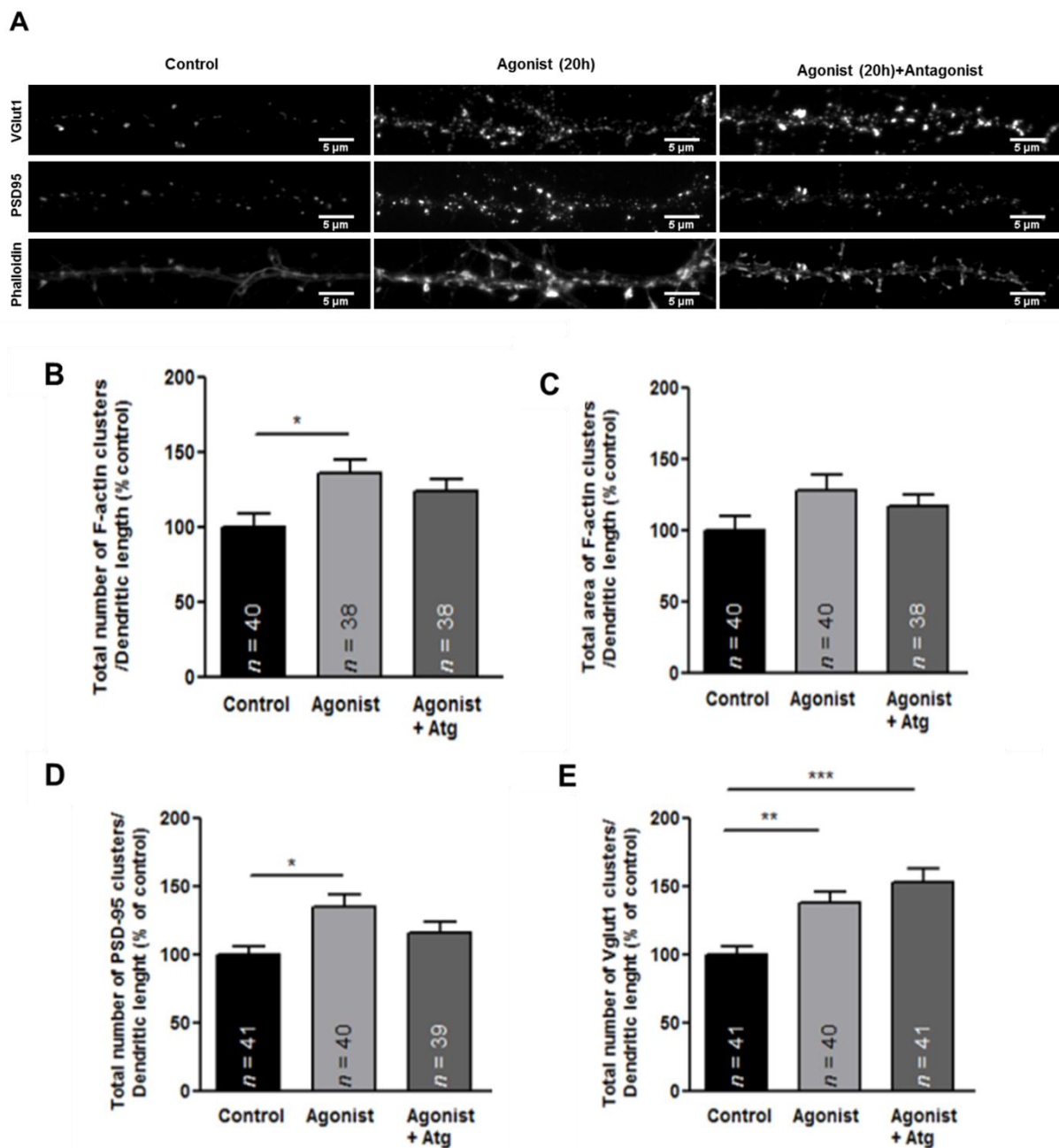**Figure 13: Effects of GHS-R1a agonist on synaptic markers in mature hippocampal neurons. A:** Representative immunofluorescence images of control, agonist-, or agonist + antagonist-treated neurons. 20 h stimuli were given at 18-19 DIV, and at 19-20 DIV neurons were stained for Vglut1, PSD-95 and F-actin (with Actin-stain 555 phalloidin). **B:** There were significant differences between conditions for F-actin cluster number (*p = 0,0211). The agonist caused an increase relative to control (*p < 0.05), which was not efficiently blocked by the antagonist, although the agonist + antagonist condition doesn't differ from control. **C:** The total area of F-actin clusters was constant. **D:** There were significant differences between conditions for PSD-95 cluster number (*p = 0,0362). The agonist caused an increase relative to control (*p < 0.05), which was not efficiently blocked by the antagonist, although the agonist + antagonist condition doesn't differ from control. **E:** There were significant differences between conditions for Vglut1 cluster number (*p < 0.0001). The agonist caused an increase relative to control (**p < 0.01). However, the antagonist did not block this effect. In fact, it produced an even greater increase relative to control (***p < 0.001). **B, C, D, E:** Data were analyzed by Kruskal-Wallis tests. Error bars represent s.e.m and *n* represents the total number of analyzed cells. Results are expressed as % of control and averaged from 4 independent experiments.

3.3. GHS-R1a activation increases cell-surface GluA1

3.3.1. The GHS-R1a agonist increases GluA1 cell-surface content in hippocampal slices

In hippocampal organotypic slices, MK-0677 increases GluA1 phosphorylation at S-831 and S-845 (Ribeiro *et al, unpublished data*), events linked to the synaptic incorporation of GluA1-containing AMPAR. Furthermore, in hippocampal neurons, MK-0677 was shown to increase this synaptic incorporation (Ribeiro *et al, unpublished data*). In order to further explore these effects, we tested the effect of MK-0677 on the cell-surface expression of GluA1 in hippocampal slices.

Acute hippocampal slices were incubated for 2 h with MK-0677 (1µM), after which cell-surface proteins were labeled with biotin and affinity-purified. The Glu1 cell-surface expression was determined, and normalized to the total GluA1 expression. We observed a significant increase in cell-surface GluA1 (fig. 14) in MK-0677-treated slices, indicating that GHS-R1a activation plays a role in cell-surface trafficking of GluA1, and that its described effects on synaptic incorporation are not (only) mediated by driving receptors already present at the surface into synaptic sites.
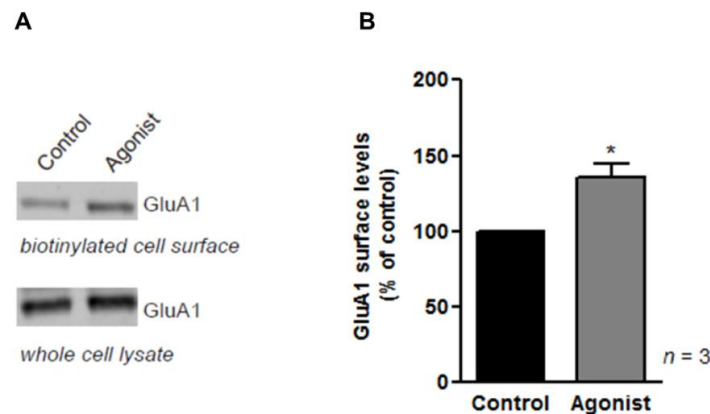
Figure 14: Effects of GHS-R1a agonist on surface GluA1 levels in hippocampal slices. Acute hippocampal slices from 8-10 week old mice were stimulated for 2h with MK-0677 and cell surface biotnylation was used to determine GluA1 surface levels. **A:** Representative western blots for biotinylated GluA1 and total GluA1. Total GluA1 levels did not alter. **B:** Mean intensity of GluA1 bands (normalized to transferrin receptor and expressed in % of control). Slice stimulation with MK-0677 led to a significant increase of surface GluA1 relative to control (*$p < 0.05$), as analyzed by a Mann-Whitney test. Error bars represent s.e.m and *n* represents the number of independent experiments.

3.3.2. Ghrelin and GluA1 trafficking *in vivo*

Next, we wanted to test whether the observed effects could be detected *in vivo* in physiologic conditions where ghrelin levels are elevated. We used a caloric restriction protocol on 8-10 week old mice to obtain high sustained ghrelin levels. The protocol consisted of 60% of normal calorie intake during 10 days, which was previously shown to cause a 4-fold increase in circulating levels of acylated ghrelin (Lutter, 2008).

We assessed the serum acylated ghrelin using an ELISA kit, but were unable to detect any increase in the ghrelin levels (Table 1), although this was probably due to the low sensitivity of the kit, combined with the fact that our samples had relatively low ghrelin concentrations. Consequently, we cannot be entirely sure that the caloric restriction protocol worked. We did, however, look at the

serum NPY levels in a few samples, and observed an increase in the serum levels of this neuropeptide in mice subjected to caloric restriction (Table 1). Considering that NPY is also an orexigenic substance and is released by NPY-AGRP neurons (stimulated by ghrelin), this seems to indicate that the caloric restriction protocol may have worked.

Table 1: Absorbance values corrected for dilution factor in the ghrelin quantification assay, and NPY concentrations in ng/ml. Paired values correspond to animals paired in the experiment.

Condition	Control	Caloric restriction
Ghrelin (Abs*dilution factor)	0,378	0,457
	0,551	0,415
	0,326	0,305
	3,455	0,404
	0,431	0,441
	0,457	0,383
	0,415	0,546
	0,173	0,436
NPY (ng/ml)	2,166	2,246
	No data	No data
	2,195	11,073
	No data	No data
	No data	No data
	4,343	4,478
	No data	No data
	No data	No data

After caloric restriction, animals were sacrificed. Animals were paired per similar weight for caloric restriction and sacrification time-course. Serum was obtained, and acute hippocampal slices were made, immediately used in cell surface biotinylation assays and probed for GluA1 (Fig. 15). We did not observe alterations in the cell surface expression of GluA1 (fig. 15A,D) or in total GluA1 expression (fig. 15B,E). Additionally, several markers linked to the synaptic targeting of AMPARs were tested: GluA1 S-831 and S-845 phosphorylation, PSD-95 content and stargazin content. None of these markers showed any change (fig. 15F-I).
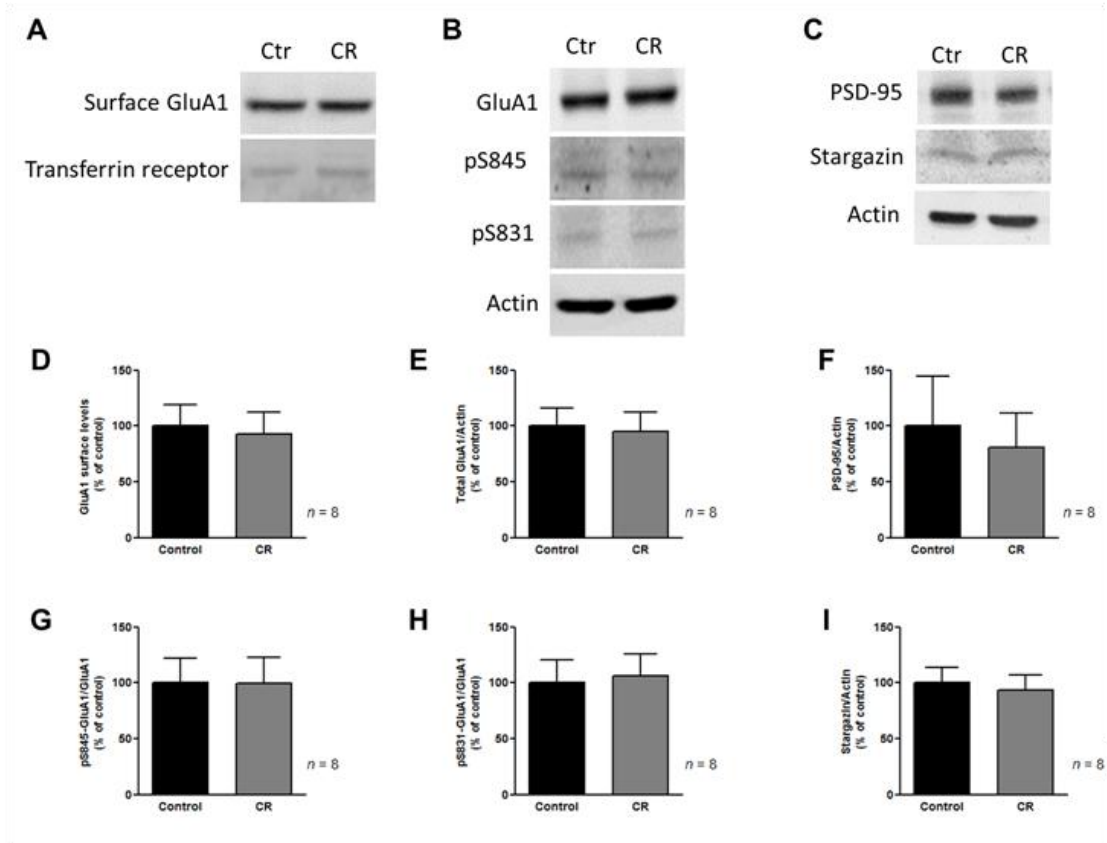
Figure 15: Effects of the caloric restriction on surface and total GluA1, as well as PSD-95 and Stargazin levels in the hippocampus. Acute hippocampal slices from 8-10 week old mice subjected to 10-day caloric restriction or fed *ad lib* (control) were used for cell surface biotinylation to determine GluA1 surface levels. **A:** Representative western blots for biotinylated GluA1 and transferrin receptor. **B:** Representative western blots for total GluA1, pS-845-GluA1, pS-831-GluA1 and actin. **C:** Representative western blots for PSD-95, stargazin and actin. **D:** Mean intensity of GluA1 bands (normalized to transferrin receptor). **E:** Mean intensity of total GluA1 bands (normalized to actin). **F:** Mean intensity of PSD-95 bands (normalized to actin). **G:** Mean intensity of pS-845- GluA1 bands (normalized to total GluA1). **H:** Mean intensity of pS-831-GluA1 bands (normalized to total GluA1). **I:** Mean intensity of PSD-95 bands (normalized to actin). **D-I:** There were no significant differences in any of the data sets, as analyzed by Mann-Whitney tests. Error bars represent s.e.m and *n* represents the number of independent experiments. Results are expressed in % of control.

Supplementary data
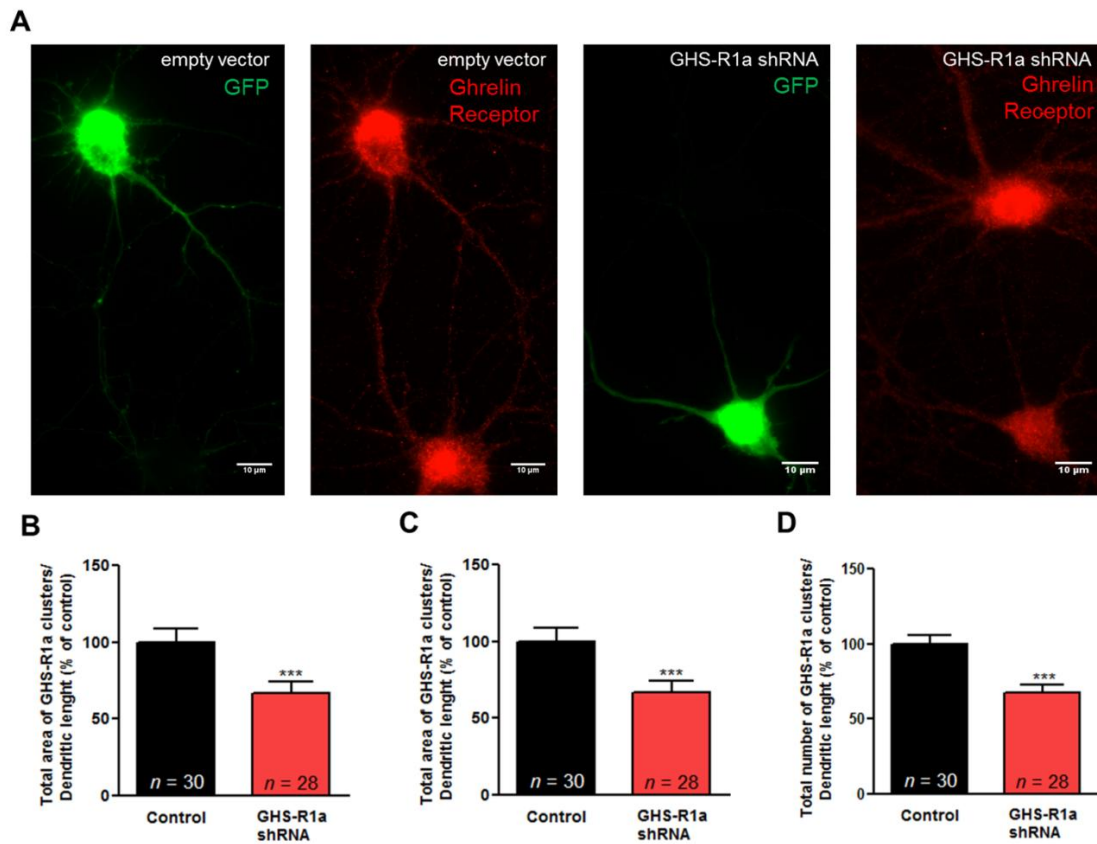
Figure S1: GHS-R1a expression after transfection with GHS-R1a shRNA in hippocampal neurons. A: Representative immunofluorescence images of control (left) and shRNA-GHS-R1a-GFP-treated (right) neurons. Cells were transfected at 7 DIV and at 15 DIV they were stained for GFP and GHS-R1a. **B:** Total intensity of GHS-R1a clusters decreased significantly ($p < 0.05$), as did total area ($p < 0.05$) **(C)** and number ($p < 0.05$) **(D),** relative to control. Data were analyzed by Mann-Whitney tests. Error bars represent s.e.m and *n* represents the total number of analyzed cells. Results are expressed as % of control and averaged from 2 independent experiments.

4. DISCUSSION

In the last decade evidence has emerged suggesting that signaling molecules involved in energy balance may also regulate hippocampal-dependent memory (Moran & Gao 2006). In fact the capacity to develop strategies to locate food sources and remembering those locations are evolutionarily important skills for survival (Moran & Gao 2006), especially when those sources are scarce. This suggests that these abilities may be tied tohormones promoting food intake, such as ghrelin, and convincing links between ghrelin and memory have in fact been found. Ghrelin was shown to increase memory retention (Carlini et al. 2004; Carlini et al. 2010; Chen et al. 2011b; Davis et al. 2010; Diano et al. 2006), promote formation of spines on hippocampal dendrites, increase LTP (0; Chen et al. 2011b) and promote the synaptic incorporation of GluA1 in hippocampal neurons (Ribeiro et al., unpublished data). Furthermore, GHS-R1a expression in the hippocampus seems to be regulated during development (Katayama et al. 2000), suggesting that it may play a role in hippocampal development.

Here, we sought to further clarify the role of GHS-R1a activity during hippocampal development; we assessed its role on dendritic arborization and on spine morphology and maturation. In addition, we explored the effects of GHS-R1a agonist MK-0677 on GluA1 surface levels. We found compelling evidence for an important role for the ghrelin receptor in regulating different aspects of hippocampal neuronal development.

The ghrelin receptor and dendritic arborization in hippocampal neurons

GHS-R1a expression suffers a consistent increase during development of hippocampal neurons, suggesting that it plays an important role in their maturation. In fact, we found that GHS-R1a knockdown in young hippocampal neurons (9-12 DIV) led to a significant decrease in arborization complexity, an effect mostly rescued by the human ghrelin receptor, which is not a target for the shRNA used. Additionally, the knockdown caused a decrease in total neurite length, though this effect was not totally rescued. These results strongly suggest that the constitutive activity of the ghrelin receptor is implicated in promoting dendritic growth and arborization during development. The fact that some of the effects were only partially rescued could mean that the human form of GHS-R1a used has a lower constitutive activity; also, even though in co-transfection cells that incorporate one of the plasmids usually incorporate the other, too, we cannot rule out the possibility that some of the visibly transfected cells in the rescue condition may have incorporated only the GHS-R1a shRNA-GFP, and not the hGHS-R1a. The fact that rescue was more efficient for distal dendrites (> 40 μm from the cell body) suggests that the ghrelin receptor may be particularly important in promoting more proximal arborization. The level of expression of the human shRNA-insensitive form of GHS-R1a may not be enough for fully rescuing normal proximal dendritic arborization.

Unexpectedly, an overexpression of GHS-R1a didn't lead to an increase in arborization complexity. This may mean that at the age studied, the downstream mechanisms of the receptor promoting arborization are saturated.

Constitutive activity of GHS-R1a is mediated by some of the same pathways as the ligand-induced activity (Holst et al. 2004; Holst et al 2003; Shi et al. 2013). Ghrelin or GHS-R1a agonist MK-0677 have been shown to improve memory performance (Carlini et al. 2004; Carlini et al. 2010; Carvajal et al 2009; Chen et al. 2011b; Diano et al. 2006) and, accordingly, enhance spine formation and LTP (Chen

et al. 2011b; Diano et al. 2006), as well as affect some of the mechanisms associated to these processes, such as CREB activation through activation of PKA (Cuellar & Isokawa 2011), actin polymerization (Berrout & Isokawa 2012), PKC activation, stargazin phosphorylation at S-239/240, phosphorylation of GluA1 at S-831 and S-845, and synaptic incorporation of GluA1 (Ribeiro et al., unpublished data). Importantly, various learning paradigms have also been shown to increase dendritic growth and/or branching in cortical pyramidal neurons (reviewed in Wong & Ghosh, 2002) and adult newborn hippocampal neurons (Tronel et al. 2010) in rats. In light of our results, the possibility arises that GHS-R1a's effect on arborization could be another link to cognitive enhancement. In fact, some of the pathways/molecules that have been shown to be involved in spine formation and/or LTP are also involved in dendritic arborization, such as Ras, ERK, PI3K, Akt, Sept7, BDNF, PKA, CREB, CaMKII, CaMKIV and actin polymerization (Qin et al. 2005; reviewed in Urbanska et al. 2008; Jaworski et al. 2005; Brand et al. 2012b; Tyler & Pozzo-Miller 2003; Caldeira et al. 2007; Sato et al. 2011; reviewed in Santos et al. 2008; Minichiello 2009; Wayman et al. 2006; Redmond et al. 2002; Fink et al. 2003). As mentioned, ghrelin or GHS-R1a agonist MK-0677 have been shown to activate PKC, activate CREB through PKA and lead to actin polymerization, and GHS-R1a's constitutive activity can be mediated through the PLC-PKC pathway or the CAMKIV-CREB pathway (Holst et al. 2004), raising the possibility that these molecules may be involved in the observed effects on arborization. Another strong candidate would be PI3K, which was shown to be activated after stimulation of hippocampal slices with the GHS-R1a agonist MK-0677 (Ribeiro et al, unpublished data), and which is implicated in dendritic arborization (Jaworski et al. 2005).

The ghrelin receptor, spine morphology and spine maturation

GHS-R1a activation has been suggested to play a role in spine formation and actin polymerization (Berrout & Isokawa 2012). We found that ghrelin receptor knockdown in young hippocampal neurons (9-12 DIV) led to a significant decrease in the number of filopodia [thought to be spine precursors (Yuste 2010)], an effect effectively rescued by hGHS-R1a, and also decreased the proportion of filopodia, while increasing the proportion of mushroom [considered to be the most stable, mature spines (reviewed in Harms & Dunaevsky, 2007)] and stubby spines, effects partially rescued by hGHS-R1a. Unexpectedly, pharmacological inhibition of the ghrelin receptor with the inverse agonist from 9 to 12 DIV didn't cause any change in the number of filopodia, but it did cause an increase in the number of mushroom spines. Possible reasons for the lack of effect of the inverse agonist could be a decay of the compound over the 3-day time course of stimulation or, as the inverse agonist causes GHS-R1a inhibition more rapidly than the knockdown, it provides a longer time for potential compensatory mechanisms to start expressing. Finally, it could be a simple consequence of the low number of cells analyzed, in which case it could be overcome by increasing the number of independent experiments.

Interestingly, stimulation with the GHS-R1a agonist MK-0677 for three days, up to 12 DIV, caused the opposite effects as the receptor knockdown, (except in case of mushroom spines, where there was no effect). Furthermore, the effect on filopodia number and stubby spine proportion was partially blocked by the antagonist, and the effect on filopodia proportion was entirely blocked by the antagonist, indicating a specific effect mediated by the ghrelin receptor. The fact that there was only a partial block in some cases could be a consequence of the small number of cells analyzed, and may become complete with an increased number of independent experiments.

Taken together, these results suggest that both the constitutive and the agonist-induced activity of the receptor play an important role in spine turnover in developing neurons, namely new spine formation and spine elimination or pruning, both of which are important during development (reviewed in Bhatt et al. 2009 and Penzes et al. 2011). It would be important to investigate which are the signaling pathways triggered by the ghrelin receptor and which could account for these effects. The PI3K and the PKC pathways have been shown to be activated in hippocampal slices upon incubation with MK-0677 (Ribeiro et al., unpublished data). In addition, the PKA pathway has been described to be activated in the hippocampus downstream of the ghrelin receptor (Cuellar & Isokawa 2011). It is plausible to speculate that the effects exerted by the ghrelin receptor on spine morphology are mediated by effects on actin dynamics. Since inhibition of NMDARs blocks LTP and active polymerization of actin (reviewed in Lamprecht & LeDoux, 2004), and NMDAR activation can lead to the formation of new spines (Lin et al. 2004), we could further speculate that the effects are activity-dependent, which is an interesting possibility if we consider that GHS-R1a activity is known to affect LTP and LTP-related events.

Since GHS-R1a activity seems to enhance spine formation, it could affect the localization of synaptic proteins. Indeed, in young hippocampal neurons, we saw that knockdown of GHS-R1a from 9 to 12 DIV caused a significant decrease in the number of both PSD-95 (a post-synaptic scaffold protein) clusters, and Vglut1 (a pre-synaptic vesicular glutamate transporter) clusters. These effects were completely rescued by hGHS-R1a, indicative of an effect mediated by the receptor, and therefore indicating that the constitutive activity of the ghrelin receptor increases the synaptic expression of PSD-95 and Vglut1 during development. Pharmacological blocking of GHS-R1a activity with the inverse agonist (9-12 DIV), however, did not cause a significant decrease in PSD-95 nor Vglut1 cluster number, although there does seem to be a tendency for decrease. The lack of statistical significance may be due to the small number of cells analyzed and may be overcome by increasing the number of independent experiments.

Considering that GHS-R1a activity increased the number of filopodia but not the number of PSD-95 containing spines, the effect of the receptor on PSD-95 expression may be a more delayed process. We can speculate that GHS-R1a activity increases filopodia formation and, in a more delayed process, increases the production of PSD-95, which may later be incorporated into spines during their maturation. Alternatively, while PSD-95 expression does seem to increase due to GHS-R1a activity, its synaptic incorporation may not; posterior synaptic incorporation may instead be dependent on activity, in which case GHS-R1a would "prime" cells for synaptic incorporation of this protein.

Roles for the ghrelin receptor in mature hippocampal neurons

In mature neurons (19-20 DIV), stimulation for 20 h with the GHS-R1a inverse agonist caused an increase in the number of PSD-95 and Vglut1 clusters, but had no effect on the number nor total area of F-actin puncta (indicatives of spine formation or enlargement, respectively). This suggests that, in mature neurons, GHS-R1a constitutive activity is important for the expression of synaptic markers, but not for spine formation. Therefore, it could be important in spine maturation or, alternatively, it could "prime" cells for the synaptic incorporation of PSD-95 and VGlut1 and maturation of spines.

Stimulation of mature neurons with MK-0677 for 1 h led to an increase in the total area of F-actin puncta, which could be an indicative of spine enlargement, and it also increased the fluorescence intensity of PSD-95 and VGlut1, suggesting it promotes spine enlargement/maturation. The effect on

PSD-95 was efficiently blocked by the antagonist, indicating a specific effect mediated by GHS-R1a, whereas the effect on VGlut1 was not blocked by the antagonist. This may mean that, in mature neurons, another, unidentified, receptor for ghrelin mediates this presynaptic effect, which is not altogether surprising, because notable differences in the binding profile among ghrelin, synthetic peptidyl (hexarelin) and non-peptidyl (MK-0677) GHSs have been found (Ong et al. 1998; Ong et al. 1998; Papotti et al. 2000; reviewed in Muccioli et al. 2007), especially in tissues that do not express GHS-R1a or express the receptor at a very low level (reviewed in Muccioli et al. 2007).

Stimulation of mature neurons with MK-0677 for 20 h led to an increase in the number of F-actin puncta, PSD-95 clusters and VGlut1 clusters, suggesting it leads to spine formation. The effect on F-actin puncta and PSD-95 clusters was partially blocked by the antagonist, but the effect on Vglut was not, again suggesting that the effect on Vglut is probably mediated by another receptor, just as in the case of 1 h stimulation. These studies should be repeated using ghrelin instead of MK-0677 in order to determine the true physiological effect, because of the different binding profiles of these 2 molecules in tissues where another receptor for ghrelin is probably present, as seems to be the case here.

Taken together, these results suggest that GHS-R1a constitutive activity is important for spine maturation, and that the agonist leads to spine maturation after 1 h, and spine formation after 20h, events that follow a similar timeline during LTP, where spine enlargement precedes spine formation (reviewed in Lin et al. 2004). Therefore, these results correlate well with previous studies showing effects of ghrelin on LTP and LTP-related events, as discussed previously.

So it appears that, during development, GHS-R1a constitutive activity is important for spine formation and expression of synaptic proteins, potentially "priming" cells for spine maturation, and the agonist induced activity is also involved in spine formation. In mature neurons GHS-R1a constitutive activity is important for the clustering of PSD-95 and Vglut, potentially "priming" cells for spine maturation/enlargement, and the agonist-induced activity is important for spine enlargement and formation, as well as the expression of synaptic markers.

Caloric restriction and hippocampal AMPA receptors

As the ghrelin receptor agonist has been shown to increase GluA1 synaptic incorporation in hippocampal neurons (Ribeiro et al., unpublished data), we wanted to know if these effects are reflected in hippocampal slices and *in vivo*. We found that treatment of hippocampal slices for 2 h with the GHS-R1a agonist significantly increased the surface content of GluA1. This also indicates that the effects of GHS-R1a on GluA1 trafficking are not (only) a simple matter of driving GluA1 already present in the membrane into synaptic sites.

We next sought to verify these effects *in vivo*, by subjecting mice to a caloric restriction protocol previously shown to cause a significant increase in circulating levels of acylated ghrelin (Lutter et al. 2008). We did not see an increase in surface GluA1 levels, or an increase in any of the other markers tested, previously shown to be enhanced in neurons stimulated with MK-0677 (Ribeiro et al, unpublished data). However, we were unable to detect an increase in plasma ghrelin levels in the caloric restricted mice compared to mice fed *ab libitum*. Therefore, we cannot be certain at this point that the caloric restriction protocol was efficient, even though NPY levels did seem to increase in the few mice that were tested. Potential normal fluctuations of ghrelin levels during the day, known to occur in humans, either eating normally (Cummings et al. 2001) or fasting (Natalucci et al. 2005), could also have affected the results. Another possible explanation could be that, *in vivo,* a learning

paradigm is needed to drive receptors into the membrane in an LTP-dependent manner, while ghrelin somehow primes for this insertion, thereby facilitating learning. In light of this possibility, it would be interesting to test these effects, subjecting mice to a learning paradigm or, at least, a more enriched environment.

Ghrelin, feeding and cognition

The ability to remember successful approaches to find food and remember locations where it was found is an important skill for survival, and may be tied to signaling molecules involved in energy balance (Moran & Gao 2006). Furthermore, caloric restriction [which results in an increase in the circulating levels of ghrelin (Lutter et al. 2008)], decreases aging-related deficiencies in cognitive processes (Witte et al. 2009), increases learning consolidation and facilitates synaptic plasticity (Fontán-Lozano et al. 2007). Conversely, high-fat and high-glucose diets [which inhibit ghrelin secretion (Beck et al. 2002; Lomenick et al. 2009), impair hippocampus-dependent synaptic plasticity and spatial memory (Stranahan et al. 2008; Wu et al. 2003). In light of this it is possible that ghrelin, which, as discussed, has been shown to have cognitive enhancing effects, could be a central player in these effects.

Here, we show that ghrelin plays a role in spine turnover, spine maturation, expression of synaptic markers and GluA1 surface expression (important for LTP), in hippocampal neurons/slices. Increases in spine number have been reported with various learning paradigms, including motor skill training and spatial memory (reviewed in Harms & Dunaevsky, 2007). On the other hand, a number of psychiatric and neurological diseases are associated with alterations in spine morphology or density (reviewed in Bhatt et al. 2009). Down's syndrome is associated with decreased spine density in the neocortex and hippocampus, and schizophrenia with decreased spine density in the neocortex. In Alzheimer's disease, the best correlate of cognitive dysfunction is thought to be the loss of synapses and, in Fragile X syndrome, which is the most frequent form of inheritable mental retardation, spines are found in much higher density (possibly due to impaired pruning) and display a more immature, long and thin form. MRI studies reveal progressive grey-matter loss before and during psychosis development in schizophrenia in late adolescence, suggesting synaptic over-pruning (reviewed in Jan & Jan, 2010). Abnormal spine morphology and number also occur in disorders such as depression (reviewed in Bhatt et al. 2009), and ghrelin elevation caused antidepressant-like effects in mice (Lutter et al. 2008).

We show that GHS-R1a constitutive activity seems to play a role in dendritic arborization, spine formation and maturation, and expression of synaptic markers. Exposure to enriched environments or training on a motor-learning task increases dendritic growth and branching in cortical pyramidal neurons (reviewed in Wong & Ghosh, 2002), and spatial learning in rats has been shown to increase the complexity of the dendritic arbor of adult newborn hippocampal neurons (Tronel et al. 2010). Several neuropathologies show abnormalities in dendritic arborization: in Alzheimer's disease, for example, there is a significant decrease in dendritic extent (Anderton et al. 1998). Increases in spine density are associated with learning paradigms, and spine abnormalities are found in various conditions (discussed above).

GHS-R1a constitutive activity may play a very important role in normal cognition, both through cerebral development and in synaptic plasticity.

Therefore, should other studies confirm the effects of GHS-R1a agonist on spines, synaptic markers and GluA1 surface expression observed in the present work, this could have serious

implications for cognitive health in conditions such as obesity or gastric bypass, both associated with decreased ghrelin levels (reviewed in Chen et al. 2009; Cummings et al. 2002). Furthermore, it would put a very different perspective on the potential of using ghrelin receptor antagonists to combat obesity and, in particular concerning the effects of the agonist in young neurons on spine formation, pruning and, probably, maturation, it could potentially have implications for cerebral development in obese children, given the lower levels of plasma ghrelin in obese humans (Cummings et al. 2002).

5. CONCLUSIONS

We show that GHS-R1a expression suffers a consistent increase during development in cultured hippocampal neurons, and that knockdown of the receptor expression decreases arborization complexity in young hippocampal neurons, strongly suggesting that GHS-R1a plays a role in dendritic arborization during neuronal development. Also, neuronal stimulation with the ghrelin receptor agonist MK-0677 caused an increase in dendritic filopodia formation, and a decrease in some types of more mature spines, whereas knockdown of the receptor caused the opposite effect and a decrease in expression of synaptic proteins. These results suggest that both the ligand-mediated and constitutive activity of the receptor are important in spine formation and maturation during development.

In more mature neurons, we show that the ghrelin receptor activation causes an increase in the expression of synaptic proteins, while pharmacological inhibition of the constitutive activity of GHS-R1a decreases the clustering of the synaptic proteins PSD-95 and Vglut. Furthermore, the ghrelin receptor agonist increases F-actin dendritic clustering, suggesting spine enlargement/maturation and/or formation. These results indicate that both the agonist-induced and constitutive activity of the receptor are important in spine formation and/or maturation in mature neurons.

We also observed that the agonist caused an increase in GluA1 surface expression in hippocampal slices, suggesting a role for ghrelin in priming AMPARs for synaptic incorporation, a probable mechanism through which it enhances LTP.

Altogether our results indicate an important role for ghrelin and its receptor in regulating morphological and functional aspects of excitatory synapses in the hippocampus.

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