Luís Filipe da Silva Ribeiro

A LINK BETWEEN METABOLIC SIGNALING AND COGNITION: THE HIPPOCAMPAL FUNCTION OF GHRELIN

Dissertação apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra, para a prestação de provas de Doutoramento em Biologia, na especialidade de Biologia Celular.

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Universidade de Coimbra

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A Link Between Metabolic Signaling and Cognition: the Hippocampal Function of Ghrelin

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Este trabalho foi realizado no Centro de Neurociências e Biologia Celular (Universidade de Coimbra), e no Centro de Biologia Molecular Severo Ochoa (Universidade Autónoma de Madrid), sob a supervisão da Professora Doutora Ana Luísa Monteiro de Carvalho (Departamento de Ciências da Vida) e co-supervisão da Professora Doutora Armanda Emanuela Castro e Santos (Faculdade de Farmácia) e financiado pela bolsa de doutoramento SFRH / BD / 47879 / 2008 da Fundação para a Ciência e Tecnologia, Portugal.

Luís Filipe da Silva Ribeiro **Coimbra, 2013**

Cover note

Cover contains a picture of *“Benefits Supervisor Sleeping”* an oil on canvas work painted by Lucian Freud, in 1995. This painting depicts an obese, naked woman lying on a couch. It is a portrait of Sue Tilley, then weighing about 127 Kg.

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Abbreviations

2-AG	2 arachidonoylglycerol
ACTH	adrenocorticotrophic hormone
AgRP	agouti gene-related peptide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type receptor
AMPK	5'-AMP-activated protein kinase
AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
ARC	arcuate nucleus
ATD	amino-terminal domain
BBB	blood-brain barrier
BCA	bicinchoninic acid
CaM	calcium/calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
CaMKK	calmodulin-dependent kinase kinase
cAMP	cyclic adenosine monophosphate
CB1R	cannabinoid receptor type 1
cGKII	cGMP-dependent kinase II
cGMP	cyclic guanosine monophosphate
CHO	chinese hamster ovary
CLAP	chymostatin, leupeptin, antipain and pepstatin
CNS	central nervous system
CRE	cAMP-responsive element
CREB	cAMP-responsive element-binding protein
CSDS	chronic social defeat stress
DAG	diacylglicerol
DG	dentate gyrus
DIV	days *in vitro*
DMH	dorsomedial hypothalamic nucleus
DRN	dorsal raphe nucleus
DTT	dithiothreitol
ECF	enhanced chemifluorescence
eNOS	endothelial nitric oxide synthase
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
GABA	4-Aminobutanoic acid
GDP	guanosine diphosphate
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRL	human ghrelin gene
Ghrl	murine ghrelin gene
GHRP	growth-hormone-releasing peptide
GHS	growth hormone secretagogue
GHS-R	growth hormone secretagogue receptor
GHS-R1a	growth hormone secretagogue receptor type 1a
GHS-R1b	growth hormone secretagogue receptor type 1b
GOAT	ghrelin O-acyltransferase
GPCR	G protein-coupled receptor
GRIP	glutamate receptor interacting protein
GTP	guanosine triphosphate
HBSS	Hank's balanced salt solution
HEK	human embryonic kidney
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HFS	high frequency stimulation
HPLC	high-performance liquid chromatography
Hz	hertz

ICV	Intracerebroventricular
iGluR	ionotropic glutamate receptors
IP_3	inositol 1,4,5-triphosphate
IPSP	inhibitory postsynaptic potential
JNK1	Jun N-terminal kinase 1
KO	knockout
LBD	ligand-binding domain
LH	lateral hypothalamus
LTD	long-term depression
LTP	long-term potentiation
M	membrane-spanning segment
MAP2	microtubule associated protein 2
MAPK	mitogen-activated protein kinase
MBOAT	membrane-bound O-acyltransferase
MCR	melanocortin receptor
mEPSC	miniature excitatory postsynaptic currents
mGluR	metabotropic glutamate-type receptor
mIPSC	miniature inhibitory postsynaptic currents
mRNA	messenger RNA
MSH	melanocyte-stimulating hormone
MTRP	motilin-related peptide
NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate-type receptor
NMU-R	neuromedin U receptor
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
NTS-R	neurotensin receptor
P	postnatal day
PBS	phosphate buffered saline
PC	prohormone convertase
PCR	polymerase chain reaction
PDZ	postsynaptic density 95/disc large/zonula occludens 1
PI3K	phosphatidylinositol 3-kinase
PICK1	protein interacting with C-kinase-1
PIP_2	phosphatidylinositol 4,5-diphosphate
PI-PLC	phosphatidylinositol-specific phospholipase C
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
POMC	pro-opiomelanocortin
PSC	postsynaptic current
PSD	postsynaptic density
PSD-95	postsynaptic density protein 95
PSP	postsynaptic potential
PVDF	polyvinylidene difluoride
PVH	paraventricular hypothalamic nucleus
RNA	ribonucleic acid
RYGB	Roux-en-Y gastric bypass
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-poliacrylamide gel electrophoresis
SSRI	selective serotonin reuptake inhibitor
TARP	transmembrane AMPA receptor regulatory protein
TBS	theta-burst stimuli
TM	transmembrane domain
TTX	tetrodotoxin
vGluT	vesicular glutamate transporter
VMH	ventromedial hypothalamic nucleus
VTA	ventral tegmental area

// Keywords

Ghrelin

Ghrelin receptor

AMPA-type glutamate receptors

Hippocampus

Long-term potentiantion

Cognition

Palavras-chave

Grelina

Receptor da grelina

Receptores do glutamato do tipo AMPA

Hipocampo

Potenciação de longa duração

Cognição

Resumo

Hormonas peptídicas como a insulina, leptina e grelina são sobretudo conhecidas pela sua função na regulação do apetite. No entanto evidências experimentais recentes têm sugerido que estes peptídeos, para além da sua função no hipotálamo como moduladores do apetite, podem também desempenhar amplos papéis na modulação das funções cerebrais.

A grelina, um peptídeo de 28 aminoácidos, é um regulador da libertação da hormona de crescimento, e um estimulador do apetite secretado a partir do estômago vazio. Observações experimentais recentes demonstram que a grelina entra no hipocampo, melhorando processos de memória dependentes desta estrutura cerebral. Deste modo, a grelina pode representar a associação molecular entre capacidades de aprendizagem e o comportamento alimentar e metabolismo energético. Esta associação poderá ser importante no sentido de assegurar a capacidade de localizar fontes de alimento, de recordar tais locais e se todo o alimento disponível foi consumido. Estas capacidades constituem importantes aptidões evolutivas que permitem a sobrevivência. Contudo, os mecanismos moleculares que estão na base dos efeitos da grelina como agente potenciador das capacidades cognitivas relacionadas com o hipocampo não se encontram ainda completamente esclarecidos.

Estudos de imunocitoquímica demonstraram que o receptor da grelina apresenta uma distribuição ponteada em neurónios do hipocampo em cultura, com uma fracção significativa do receptor a localizar-se em sinapses glutamatérgicas. Além disso, o receptor da grelina foi identificado em fracções sinápticas purificadas a partir de hipocampo de ratos adultos. Este padrão de localização sináptica do receptor da grelina sugere uma possível função da grelina na modulação da transmissão sináptica excitatória. Para testar esta hipótese começámos por tratar culturas de neurónios do hipocampo com um agonista do receptor da grelina (MK-0677), e realizámos uma análise de imunofluorescência quantitativa com o objectivo de avaliar a expressão superficial sináptica de GluA1, uma subunidade dos receptores de glutamato do tipo AMPA (AMPARs). Recorrendo a esta abordagem experimental observámos que o tratamento com MK-0677 levou a um aumento dos níveis de GluA1 à superfície dos neurónios e na sinapse.

Com o objectivo de determinar directamente se a grelina induz a inserção de novos AMPARs na sinapse, a subunidade GluA1 ligada a GFP (GluA1-GFP) foi expressa em neurónios da região CA1 em fatias organotípicas do hipocampo. Observámos que o tratamento com grelina, ou com o agonista do receptor da grelina, aumentam a inserção sináptica de AMPARs homoméricos contendo GluA1-GFP. Estes resultados sugerem fortemente que a activação do receptor da grelina induz a inserção sináptica de AMPARs que contêm a

subunidade GluA1. Com o objectivo de verificar se a activação do receptor da grelina produz uma alteração funcional na sinapse CA3-CA1 do hipocampo, fatias organotípicas foram incubadas com o agonista, e em seguida foram realizados registos electrofisiológicos. Após a indução de respostas sinápticas a partir de neurónios da região CA1, os rácios entre as correntes mediadas por receptores do tipo AMPA/NMDA e NMDA/GABA foram calculados. Observámos que o rácio entre as correntes AMPA/NMDA aumentou após o tratamento com MK-0677, enquanto o rácio entre as correntes NMDA/GABA não se alterou. No seu conjunto, estes resultados demonstram que a activação do receptor da grelina aumenta a transmissão sináptica mediada por receptores do tipo AMPA no hipocampo, através da inserção sináptica de AMPARs que contêm GluA1.

Observámos também que o tratamento com MK-0677 aumenta dramaticamente a expressão da potenciação de longa duração (LTP) dependente de receptores de glutamato do tipo NMDA na sinapse CA3-CA1 do hipocampo. Além disso, verificámos que após indução de LTP químico (um modelo de LTP para neurónios do hipocampo em cultura), em neurónios previamente sujeitos ao agonista, existe um aumento do endereçamento sináptico de receptores endógenos do tipo AMPA contendo a subunidade GluA1. Este resultado experimental demonstra que a activação do receptor da grelina aumenta a inserção sináptica de AMPARs, o que leva à facilitação da expressão de eventos relacionados com LTP. Uma vez que alterações na força sináptica são consideradas como sendo o alvo celular para o armazenamento de memória no cérebro, o efeito da activação do receptor da grelina na plasticidade sináptica do hipocampo poderá justificar a acção da grelina como um agente potenciador da cognição.

As alterações causadas pela activação do receptor da grelina, no tráfego de receptores do tipo AMPA e que alteram as características funcionais da transmissão sináptica excitatória e a expressão de LTP, foram acompanhadas por aumentos quer na fosforilação de GluA1, quer na fosforilação de uma proteína associada aos receptores do tipo AMPA, designada por stargazina. Além disso, observámos também a activação das vias de sinalização responsáveis pela fosforilação destes alvos moleculares, os quais se sabe serem necessários para a indução de inserção sináptica de AMPARs e expressão de LTP.

Finalmente, obtivemos evidências que indicam que a função do receptor da grelina na regulação da transmissão sináptica mediada por receptores do tipo AMPA no hipocampo é regulada ao longo do desenvolvimento. Enquanto em fatias organotípicas do hipocampo mais jovens as acções mediadas pelo receptor são dependentes do ligando, em fatias mais velhas é a actividade constitutiva do receptor da grelina que regula a transmissão mediada por AMPARs.

Em conclusão as observações experimentais aqui descritas sugerem que as propriedades da grelina como agente potenciador da cognição são mediadas pela sua capacidade de aumentar a inserção sináptica de receptores do tipo AMPA que contêm GluA1 no hipocampo. Este processo celular é um dos mais bem caracterizados como sendo necessário para as alterações de longa duração na força sináptica que estão na base da formação de memória e aprendizagem.

Abstract

Peptide hormones such as insulin, leptin and ghrelin are well known for their role in the regulation of appetite. Recent evidence suggests that these peptides, in addition to acting on the hypothalamus to modulate food intake, may play wider roles in modulating brain functions.

Ghrelin, a 28 amino acids peptide, is a regulator of growth hormone release, and an appetite-stimulating hormone, secreted from the empty stomach. Recent data show that ghrelin enters the hippocampus, enhancing hippocampal-dependent memory processes. Thus, ghrelin may represent the molecular link between learning capabilities associated to feeding behavior and energy metabolism, ensuring the ability to locate food sources, remember those locations, and recall whether all available food has been consumed, which are evolutionarily important skills for survival. However the molecular mechanisms that underlie the effects of ghrelin as a hippocampal cognitive enhancer are still not completely understood.

Immunocytochemistry analysis showed that the ghrelin receptor presents a punctate distribution in hippocampal cultured neurons, with a significant population of ghrelin receptors localized to glutamatergic synapses. Moreover, the ghrelin receptor was found in synaptic fractions obtained from adult rat hippocampi. This synaptic localization of the ghrelin receptor suggests a possible involvement of ghrelin in modulating excitatory synaptic transmission. To start testing this hypothesis hippocampal cultured neurons were treated with a ghrelin receptor agonist (MK-0677) and quantitative immunofluorescence analysis was performed to analyze the synaptic cell surface expression of GluA1, a subunit of the AMPA-type glutamate receptors (AMPARs). Treatment with MK-0677 led to an increase in cell surface GluA1 levels colocalized with an excitatory synaptic marker.

To directly determine whether ghrelin induces the delivery of new AMPARs into synapses, the GFP-tagged GluA1 subunit (GluA1-GFP) was expressed in CA1 neurons in organotypic hippocampal slice cultures. Slice treatment with ghrelin or with the ghrelin receptor agonist increased the synaptic delivery of homomeric GluA1-GFP AMPARs in activity-dependent manner. These data strongly suggest that activation of the orexigenic ghrelin hormone receptor induces synaptic delivery of GluA1-containing AMPARs. To test whether ghrelin receptor activation produces a functional change at excitatory CA3-CA1 synapse, organotypic hippocampal slices were treated with the ghrelin receptor agonist and electrophysiological recordings were performed. Synaptic responses were evoked, and the ratios between AMPA/NMDA and NMDA/GABA currents recorded from CA1 neurons were calculated. The AMPA/NMDA ratio of synaptic responses significantly increased after MK-0677 treatment, while the NMDA/GABA ratio was found to be unaltered. Altogether, these results

suggest that the ghrelin receptor activation increases AMPARs-mediated synaptic transmission in the hippocampus, by inducing delivery of GluA1-containing AMPARs receptors into synapses.

Moreover, we found that MK-0677 treatment dramatically enhanced NMDARs-dependent long-term potentiation (LTP) expression in the hippocampal CA3-CA1 synapse. Agonist application also increased the synaptic trafficking of endogenous GluA1-containing AMPARs in hippocampal cultured neurons upon chemical LTP induction (a model for LTP in hippocampal cultures). These findings suggest that ghrelin receptor activation increases the AMPARs delivery to synapses, facilitating the expression of LTP-like events. Given that changes in synaptic strength are considered the cellular substrate for memory storage in the brain, the effect of ghrelin receptor activation on hippocampal synaptic plasticity may underlie the cognition enhancing properties of ghrelin.

The changes in AMPARs trafficking, and consequent functional alterations in hippocampal excitatory synaptic transmission and LTP expression, triggered by ghrelin receptor activation, were accompanied by increases in the phosphorylation of GluA1, as well as in the AMPARs-associated protein stargazin. Moreover, we observed an increase in the activation of the signalling pathways responsible for the phosphorylation of these molecular targets, which are known to be required for the induction of AMPARs synaptic trafficking, and for LTP expression.

Finally, we obtained evidence for a developmentally regulated function of ghrelin receptor activation on AMPARs-mediated synaptic transmission. Whereas in young hippocampal slices the action of the ghrelin receptor is dependent on ligand-dependent activation, in more mature hippocampal slices the constitutive activity of the ghrelin receptor regulates AMPAR-mediated transmission.

In conclusion, these findings point to a scenario in which the cognitive enhancing properties of ghrelin are likely mediated by its potential to increase the synaptic trafficking of GluA1-containing AMPARs, one of the most well characterized cellular processes required for the long lasting changes in synaptic strength underlying learning and memory formation.

Chapter 1

Introduction

Ghrelin

A Novel Hormone from the Stomach

In December 1999, an endogenous ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a or ghrelin receptor) was first reported (Kojima et al., 1999). It was named *ghrelin* (Figure 1), based on “ghre”, a word root in Proto-Indo-European languages for “growth”, in reference to its ability to stimulate growth hormone (GH) release (reviewed in Kojima, 2008). Ghrelin is a 28-amino acid peptide originally purified from the rat stomach (Kojima et al., 1999) in which the third N-terminus amino acid, a serine residue (Ser^3), is post-translational modified with a 8-carbon acyl group (octanoylation). In other words, the hydrogen atom of the hydroxyl of Ser^3 is replaced by a hydrophobic moiety, $C_7H_{15}CO$ (octanoyl group). This post-translation modification is essential for ghrelin’s activity and so far unique to this peptide, as no other naturally occurring peptides have been shown to have this acyl group as a post-translation modification.

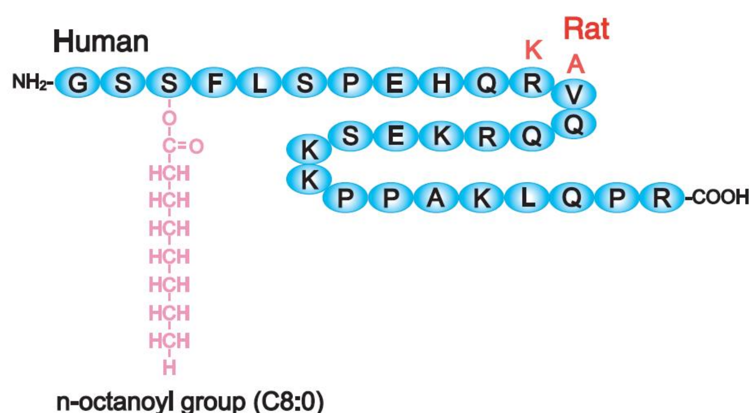


Figure 1. Structure of human and rat ghrelin. Both human and rat ghrelin are 28-amino acid peptides, in which Ser^3 is modified by a fatty acid, *n*-octanoic acid. This modification is essential for ghrelin’s activity. [Reproduced from (Kojima and Kangawa, 2005)].

The discovery of ghrelin is the result of a typical story of reverse pharmacology, which started with the discovery of synthetic, non-natural growth-hormone-releasing peptides (GHRP), also designated growth hormone secretagogues (GHS), now classified as ghrelin mimetics. GHSs are synthetic compounds that are potent stimulators of GH release, working through a G protein-coupled receptor (GPCR), the GHS-receptor (GHS-R) (Howard et al., 1996; Pong et al., 1996; Korbonits et al., 1999). In their pioneering work, Bowers and co-workers demonstrated that small synthetic peptides derived from the natural opiate met-enkephalin specifically release GH *in vitro* (Bowers et al., 1980). Although the activity of early GHSs was very weak, their discovery led to the synthesis of many peptide and non-peptide compounds with more potent GH-releasing activity. Particularly, GHRP (growth hormone-releasing peptide)-6, GHRP-1, GHRP-2 (KP-102), hexarelin, ipamorelin (peptide compounds) and L-692,429, L-163,191 (MK-0677), NN703 (non-peptide compounds) were developed, and showed to have potent GH-

releasing activity when administered parenterally or orally (Bowers et al., 1984, 1990; Smith et al., 1993, 1997, 2004, 2005; Cheng et al., 1993; Deghenghi et al., 1994; Patchett et al., 1995; Chapman et al., 1996; Camanni et al., 1998; Smith, 2005). Several GHRP/GHS compounds were studied clinically, but none has reached the market (Smith et al., 2004, 2005; Isidro and Cordido, 2006). In particular, the spiroperidine derivative MK-0677 was subjected to clinical trials, since it retained sufficient activity even when orally administered (Patchett et al., 1995; Chapman et al., 1996; Thorner et al., 1997). These compounds bind to specific sites in the cerebral cortex, hippocampus, medulla oblongata, choroid plexus, with the greatest density of binding sites in the hypothalamus and pituitary gland (Sethumadhavan et al., 1991; Howard et al., 1996; Muccioli et al., 1998). GH is controlled by many factors, in particular by two hypothalamic neuropeptides; GH release is stimulated by hypothalamic GH-releasing hormone (GHRH) and inhibited by somatostatin (Müller et al., 1999; Anderson et al., 2004). GHSs activate a third independent pathway regulating GH release. GHRH promotes GH secretion from GH-secreting cells in the anterior pituitary, acting on the GHRH receptor to increase intracellular cyclic adenosine monophosphate (cAMP) (Blake and Smith, 1991; Cheng et al., 1991; Akman et al., 1993; Popovic et al., 1996). GHSs act on a different receptor on GH-secreting cells in the anterior pituitary, increasing the intracellular calcium (Ca^{2+}) concentration via an inositol 1,4,5-triphosphate (IP_3) signal transduction pathway. The GHS receptor was in fact cloned in 1996, as a typical G-coupled protein receptor, based on the findings that GHSs stimulate phospholipase C, resulting in an increase in IP_3 and intracellular Ca^{2+} (Howard et al., 1996). It was found that this receptor is mainly expressed in the hypothalamus and pituitary gland and it became known as the type 1a GHS receptor (GHS-R1a) (Howard et al., 1996).

GHS-R1a was for some time an example of an orphan GPCR, that is, a GPCR with no known natural bioactive ligand, although it was postulated that there must exist an endogenous ligand that binds to GHS-R carrying out similar functions to GHSs. The orphan-receptor based strategy to identify endogenous ligands (reviewed in Civelli, 1998) consists, first, in establishing a cell line expressing an orphan receptor. Then, a peptide extract is applied to the cell and a second messenger response is measured. If a target orphan receptor is functionally expressed on the cell surface and the extract contains the endogenous ligand that can activate the receptor, the second messenger response, for example monitored by the levels of cAMP or intracellular Ca^{2+} concentration, will decrease or increase. Using this assay system, the endogenous ligand can be purified through several chromatographic steps. This reserve pharmacology strategy led to recognition and characterization, for instance, of the endogenous opiates and endocannabinoids (Olson et al., 1979; Elphick and Egertová, 2001). Based on this strategy, orphan receptors represent important new tools for the discovery of novel bioactive molecules and in drug development (Civelli et al., 2001; Howard et al., 2001; Wise et al., 2004).

Finally, in 1999 Kojima and co-workers had success in the purification and identification of the endogenous ligand for the GHS-R (Kojima et al., 1999). In opposition to the expected,

since the known sites of GHS-R expression at that time were the pituitary gland and the hypothalamus (Howard et al., 1996), ghrelin was purified from stomach extracts by monitoring Ca^{2+} concentration changes in a Chinese hamster ovary (CHO) cell line stably transfected with human GHS-R1a (Kojima et al., 1999). Later, after the first evidence of a positive effect on appetite mediated by some GHSs (Locke et al., 1995; Okada et al., 1996; Shibasaki et al., 1998; Torsello et al., 1998), the function of ghrelin as a peripheral modulator of food intake and body weight was established (Tschöp et al., 2000). It was found that rat serum ghrelin concentrations were increased by fasting and were reduced by re-feeding, suggesting that ghrelin, in addition to its role in regulating GH secretion, signals the hypothalamus when an increase in metabolic efficiency is necessary (Tschöp et al., 2000). These were the starting days of the characterization of ghrelin as a crucial orexigenic peripheral hormone.

Ghrelin Family Members

In 2000 Tomasetto and co-workers identified a stomach-derived mRNA coding for a protein, specifically expressed by enteroendocrine cells, with sequence similarity with prepro-motilin, the precursor of the motilin hormone and the motilin-associated peptide (Tomasetto et al., 2000). This hormone peptide was named prepro-motilin-related peptide, which after post-translational maturation originates the motilin-related peptide (MTRP) (m46) and MTRP-associated peptide (Tomasetto et al., 2000). This peptide later turned out to be identical to ghrelin (95% identity between the rat ghrelin precursor and the mouse m46 protein precursor), although the post-translational modification with the acyl group was not recognized (Coulie and Miller, 2001; del Rincon et al., 2001). In this study, the peptide was expressed by transfecting COS-1 cells and submitted to protein sequencing. In another study Kojima and co-workers analysed ghrelin purified from stomach by electrospray ionization mass spectrometry and identified a $C_7H_{15}CO$ moiety bound to the hydroxyl group in Ser^3 (Kojima et al., 1999). In fact, ghrelin and the gastrointestinal peptide hormone motilin share several common features. Motilin and ghrelin precursors share almost 50% similarity in their amino acid sequences (Tomasetto et al., 2000), and the motilin receptor is the closest relative of the ghrelin receptor (with 52% amino acid sequence identical) (Feighner et al., 1999). The ghrelin receptor and motilin receptor belong to the same family of GPCRs. Additionally, ghrelin and motilin play similar roles in the stomach (reviewed in Ohno et al., 2010), as well as on food intake and growth hormone secretion (Folwaczny et al., 2001). Based upon their structural and functional similarities, the two peptides form a peptide family (motilin-ghrelin peptide family), linking endocrine control of energy balance and growth with regulation of gastrointestinal mobility, and may have evolved from a common ancestral system (Folwaczny et al., 2001).

The ghrelin gene

The human ghrelin gene (*GHRL*) (Figure 2) spans approximately 5 kilobases, is located on the short arm of choromossome 3 (3p25-26) and contains four prepro-ghrelin (or ghrelin precursor)-coding exons (1–4 exons) (Wajnrajch et al., 2000) and one non-coding first exon (20 bp exon 0, which encodes part of the 5' untranslated region) (Kanamoto et al., 2004; Nakai et al., 2004). The murine ghrelin gene (*Ghrl*) maps to chromosome 6 and has a gene structure that is almost identical to the human gene (Tanaka et al., 2001). Interestingly, the mouse and rat ghrelin genes also contain a non-coding 19 bp exon 0, which is highly homologous to human exon 0 (Tanaka et al., 2001). The described human cDNA codes for a 117 amino acid long prepro-ghrelin (Kojima et al., 1999), which contains a 23 amino acid signal peptide and 94 amino acid pro-ghrelin (with 28 amino acid corresponding to the mature ghrelin and a 66 amino acid tail – C-terminal polypeptide or C-ghrelin). During prepro-ghrelin processing the 23 amino acid secretion-signal peptide is cleaved from the 117 amino acid precursor, resulting in the 94 amino acid pro-ghrelin peptide (amino acids 24–117). This pro-ghrelin peptide is then cleaved further and gives rise to the 28 amino acid ghrelin peptide, (amino acids 24–51), and the 66 amino acid polypeptide C-ghrelin (Zhu et al., 2006) (Figure 3). The prepro-ghrelin signal peptide is encoded by exon 1, the coding sequence of the 28 amino acid ghrelin peptide hormone is encoded by parts of exons 1 and 2, while part of exon 2 and exons 3 and 4 encode the C-terminal polypeptide.

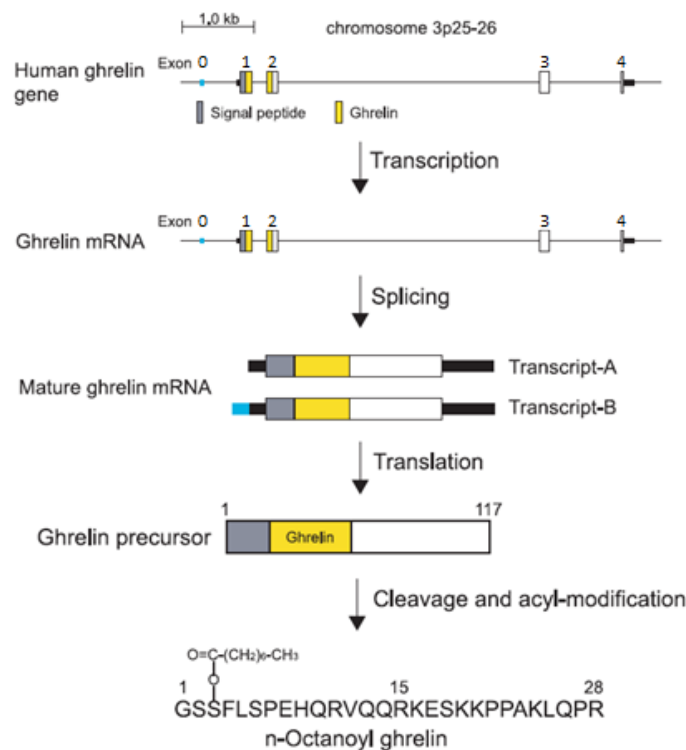


Figure 2. From the human ghrelin gene to an active peptide. The human ghrelin gene comprises five exons. The first exon (exon 0) encodes the 5'-untranslated region and is very short. cDNA analyses of human ghrelin have revealed transcript A, an alternative splicing product from exon 1 to exon 4, and transcript B (containing also the exon 0). This mRNA is translated into a 117-amino acid ghrelin precursor (prepro-ghrelin). Protease cleavage and acyl-modification of the ghrelin precursor result in the production of a 28-amino-acid-long active acyl-modified ghrelin peptide. [Adapted from (Kojima and Kangawa, 2005)].

The 5'-flanking region of the human ghrelin gene contains a TATA box-like sequence, as well as several putative binding sites for several transcription factors, such as AP-2, USF1, USF2, PEA-3, Myb, NF-IL6, hepatocyte nuclear factor-5 and NF-B, and half-sites for estrogen and glucocorticoid response elements (Kishimoto et al., 2003; Kanamoto et al., 2004), indicating that these transcription factors may regulate ghrelin expression. However, evidence for the direct regulation of these transcriptional factors on ghrelin gene expression is still largely missing. While neither mutation nor deletion of the TATA box-like element decreased the promoter activity (Kishimoto et al., 2003), suggesting that this is not functional, the destruction or site-directed mutagenesis of E-box consensus sequences (bound by USF1 and USF2 transcription factors) decreased the promoter activity (Kanamoto et al., 2004). The basal transcription activity of the human core promoter seems to require a sequence downstream of the distal non-coding exon 0 and a proximal sequence of intron 1 (Wei et al., 2005).

Regulation of the ghrelin gene expression

Among determinants of ghrelin secretion the most important appear to be glucose, insulin, leptin, somatostatin, growth hormone, glucagon, thyroid hormones, melatonin, as well

as the parasympathetic nervous system, among others (reviewed in Korbonits et al., 2004 and Yin et al., 2009).

Of particular interest, Kishimoto and co-workers showed that glucagon and its second messeger cAMP increase promoter activity of the ghrelin gene in the stomach-derived ECC10 cell line (Kishimoto et al., 2003). This increase, mediated by glucagon, was also confirmed in another stomach-derived cell line (AGS cells) (Wei et al., 2005). Additionally, Wei and co-workers demonstrated that glucagon treatment increases stomach ghrelin mRNA levels in rats (Wei et al., 2005), suggesting that in fasting conditions the increase in ghrelin levels (e.g., Tschöp et al., 2000) may be related with an increase in glucagon, which is consistent with the increase in systemic glucagon levels observed after food restriction (Seino et al., 1980). Moreover, glucagon receptor is present in endocrine cells in gastric mucosa (Katayama et al., 2007), plasma ghrelin concentration rises after administration of glucagon in rats (Katayama et al., 2007), and ghrelin released from the rat stomach is augmented by glucagon perfusion (Kamegai et al., 2004). However, the mechanism by which glucagon up-regulates ghrelin gene expression remains unclear because no glucagon-related binding sites in the promoter region of ghrelin gene have been identified.

Leptin also seems to regulate the ghrelin gene expression, although controversial data have emerged. Leptin dose-dependently inhibited ghrelin mRNA expression in *vitro*, in stomach tissue culture prepared from rat (Zhao et al., 2008). Infusion of leptin directly into the rat stomach dose-dependently decreased ghrelin secretion from isolated stomach (Kamegai et al., 2004), but the intraperitoneal injection of leptin, for 5 consecutive days, increased ghrelin mRNA levels in the gastric fundus in mice (Toshinai et al., 2001). In humans, no effect of leptin administration on circulating levels of ghrelin has been found (Chan et al., 2004). Therefore, leptin does not seem to be a major regulator of the ghrelin gene expression. Later in this chapter (section: "*Regulation of ghrelin release*"), the regulation of ghrelin gene expression and secretion will be further explored, in particular its regulation by the physiological status (reviewed in Yin et al., 2009).

Alternative splicing of the ghrelin gene

It is not surprising that the ghrelin gene may give rise to several alternative transcripts, as has been show for other genes with a variety of cellular functions. Protein diversity is increased by alternative use of promoters (e.g., different transcriptional initiation sites), splice sites, translational start sites and translational terminations codons (reviewed in Black, 2003). There are two different transcriptional initiation sites in the human ghrelin gene, one located at –80 and the other at –555 relative to the ATG initiation code, resulting in two distinct mRNA transcripts, respectively transcript-A (shorter transcript, lacking the codon 0) and transcript-B (longer transcript) (Kanamoto et al., 2004) (Figure 2). Quantitative real-time PCR analysis suggested that both transcripts are equally expressed in the human stomach, whereas in a

human medullary thyroid carcinoma (TT) cell line the transcript A was mostly expressed (Kanamoto et al., 2004).

In rat and mouse there are two types of ghrelin precursors: the previously reported 117-amino acid precursor (prepro-ghrelin) (Kojima et al., 1999), which contains the 28 amino-acid ghrelin, and a 116-amino acid precursor, which contains the des-Gln^{14}-ghrelin [a 27-amino acid peptide homologous to ghrelin, except that one glutamine (14th Gln of ghrelin) is missing] (Hosoda et al., 2000b; Tanaka et al., 2001) (Figure 5). Des-Gln14-ghrelin is modified at its third serine by the octanoyl group in a manner similar to ghrelin (Kojima et al., 1999). This post-translational modification is also needed to activate GHS-R1a and des-Gln^{14}-ghrelin efficiently induces intracellular Ca^{2+} concentration increases in cells expressing GHS-R1a. When des-Gln^{14}-ghrelin is intravenously injected into rats, it increases the GH concentration in the plasma, similarly to ghrelin injection (Hosoda et al., 2000b). Ghrelin and des-Gln14-ghrelin are the result of alternative splicing (Hosoda et al., 2000b). The ratios observed between the two precursor populations, prepro-ghrelin and prepro-des-Gln^{14}-ghrelin, were 5 to 1 in rat stomach, and 6 to 5 in mouse stomach (Hosoda et al., 2000b; Tanaka et al., 2001). In human, nearly all of the cDNA clones isolated from stomach encode the prepro-ghrelin precursor, a few cDNA clones encode the prepro-des-Gln^{14}-ghrelin precursor (Hosoda et al., 2003). However, the amount of des-Gln^{14}-ghrelin from the human stomach is negligible (Hosoda et al., 2003).

Additionally, a exon 3-deleted prepro-ghrelin mRNA transcript has also been described in breast and prostate cancer in humans (Jeffery et al., 2005b; Yeh et al., 2005) and in mice (Jeffery et al., 2005a). The exclusion of exon 3 from prepro-ghrelin mRNA transcript results in a cDNA frameshift and the generation of a premature stop codon. When translated, this transcript encodes a 91 amino acid prepro-ghrelin peptide in humans and an 86 amino acid prepro-ghrelin peptide in mice. While still producing functional, mature ghrelin, it also produces a C-terminal polypeptide (Δ3 C-ghrelin), which gives rise to a novel, 16 amino acid Δ3 C-terminal peptide in humans and 11 amino acid Δ3 C-terminal peptide in mice (Jeffery et al., 2005a, 2005b; Yeh et al., 2005). This Δ3 C-terminal peptide begins with a potential dibasic proteolytic cleavage site (Arg-Arg) (Jeffery et al., 2005a; Yeh et al., 2005). It remains to be investigated whether the Δ3 C-ghrelin or its proteolytic product Δ3 C-terminal peptide circulate in plasma, as does the C-ghrelin derived from wild-type (full-length) pro-ghrelin (Pemberton et al., 2003). Although the functions of the Δ3 C-terminal peptide remain unknown, it is present in a number of tissues in the mouse (Jeffery et al., 2005a) and it is upregulated in human prostate and breast cancer (Jeffery et al., 2005b; Yeh et al., 2005), strongly suggesting that the peptide is functional. These two splicing variants are the only that have been experimentally proven to be translated into peptides. Several additional alternative splicing transcripts have been suggested (reviewed in Seim et al., 2009), however, further studies are required to examine the relative abundance, tissue distribution, coding potential and physiological significance of these transcripts.

Prepro-ghrelin processing through alternative splicing (as indicated before) and the use of alternative post-translational cleavage sites (Hosoda et al., 2000a, 2003; Pemberton et al., 2003; Zhang et al., 2005) result in the synthesis of novel peptides that are biologically active and can act through different receptors and have different functions. In accordance with this, prepro-ghrelin may also be described as a polyhormone, similarly to parathyroid hormone (Nemeth, 2006), which indicates that their derived biologically active peptides are originated from the same precursor prepro-gene. These peptides, which could represent a previously hidden layer of biological diversity, have been termed crypteins (from cryptein, ‘‘to hide’’ in Greek). Crypteins may be functionally similar to their parent molecules or have novel functions (Autelitano et al., 2006; Pimenta and Lebrun, 2007; Ueki et al., 2007). A well-described example is pro-opiomelanocortin (POMC), a prepro-hormone that is enzymatically cleaved to form the appetite-suppressing melanocyte-stimulating hormones, α-MSH and β-MSH, and the appetite-stimulating hormone, β-endorphin, as well as a large number of other bioactive crypteins (reviewed in Raffin-Sanson et al., 2003).

Post-translational modification of the prepro-ghrelin peptide

Processing of prepro-ghrelin to mature ghrelin

The polypeptide prepro-ghrelin (117 amino acids) contains a signal peptide of 23 amino acids at the N-terminal, which is first cleaved by a putative signal peptidase resulting in pro-ghrelin (amino acids 24–117) with 94 amino acids (reviewed in Garg, 2007) (Figure 3). The signal peptide is enzymatically cleaved off from the prepro-ghrelin inside the endoplasmic reticulum (ER), and generally is destroyed at this point. The enzymes responsible for further proteolytic cleavage of pro-ghrelin to the final products remain largely unknown. However, it was suggested that prohormone convertase 1/3 (PC1/3) is the endoprotease responsible for the conversion of pro-ghrelin to ghrelin (Zhu et al., 2006). It was observed that ghrelin was absent in PC1/3 null mouse stomachs, while the pro-ghrelin was present in the PC1/3 null samples, when assessed by Western blot (Zhu et al., 2006). Additionally, ghrelin is co-localized with PC1/3 in the ghrelin-producing cells of the stomach and the ghrelin gene expression is upregulated in PC1/3 null mice (Zhu et al., 2006). This study also suggested that octanoylation of ghrelin does not require prior processing of the peptide, since acylated pro-ghrelin was found in the PC1/3 null mice stomach. In addition, it is highly likely that PC1/3 is involved in the processing of obestatin (peptide derived from the C-terminal polypeptide part of pro-ghrelin, a ghrelin gene-derived cryptein peptide, Zhang et al., 2005), since only the full length precursor (pro-ghrelin), and not any intermediate products were detected in PC1/3 nulls. Later, this *in vivo* study was confirmed by overexpression of human pro-ghrelin in *E.coli* and posterior *in vitro* cleavage using mouse PC1 (Ozawa et al., 2007). Ozawa and co-workers additionally suggest that human PC7 and human furin may also be involved in ghrelin processing (Ozawa et al., 2007). A product with a size corresponding to obestatin was never found with *in vitro* digestion of pro-ghrelin (Ozawa et al., 2007), in disagreement to Zhu and co-workers (Zhu et al., 2006), suggesting that the

production of this peptide might require the activity of other proteases, perhaps from blood, since the accumulation of C-terminal fragments of ghrelin in plasma has been reported (Pemberton et al., 2003). In conclusion, the detailed mechanism on how obestatin is processed remains to be explored.

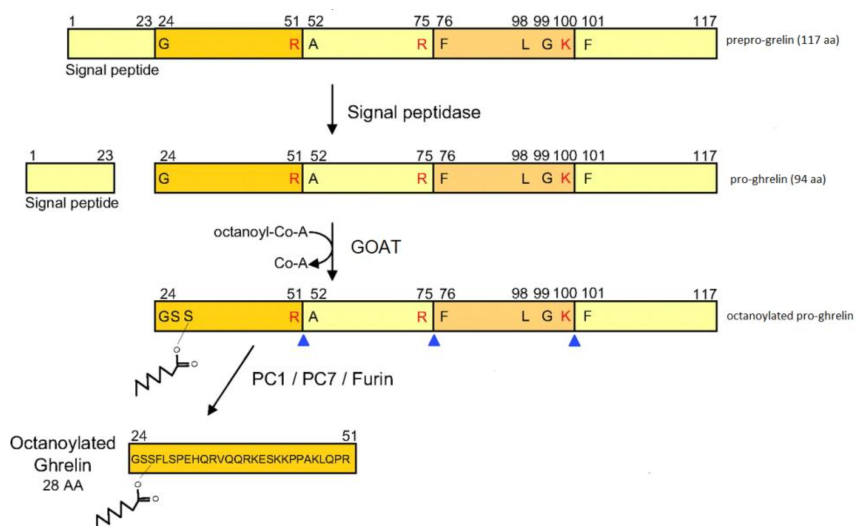


Figure 3. Post-translational processing of prepro-ghrelin to mature ghrelin. This polypeptide contains a signal peptide of 23 amino acids at the amino terminus, which is first cleaved by a putative signal peptidase resulting in pro-ghrelin with 94 amino acids. Further processing of pro-ghrelin to ghrelin involves proteolytic cleavage by prohormone convertases (PCs). These enzymes identify the monobasic residues arginine and lysine (*red*). The *blue triangles* indicate the sites of proteolytic cleavage. The PCs cleave pro-ghrelin between arginine 51 and alanine 52. Arginine 51 is preceded by proline at position 50, which may take this site a preferred site for PC cleavage. Ghrelin is also known to be acylated (preferably by octanoic acid) at the serine at position 3. This acylation may occur before the cleavage of pro-ghrelin to ghrelin and is processed by the O-acyltransferases (GOAT). Obestatin results from proteolytic cleavage of pro-ghrelin at two cleavage sites, between arginine 75 and phenylalanine 76, and between lysine 100 and phenylalanine 101. It is proposed that arginine 75 and lysine 100 predict the monobasic recognition sites for an unknown protease besides the known PCs. [Adapted from (Garg, 2007)].

Octanoylation of ghrelin

Analysis by mass spectroscopy revealed that serine-3 (Ser^3) of ghrelin is modified by acylation with an octanoyl group, which is required for growth hormone releasing activity and for binding to its receptor GHS-R1a (Kojima et al., 1999). Ser^3 is conserved in mammals, birds, and fishes (Figure 4). In ghrelin of the bullfrog, Ser^3 is replaced by threonine, but this residue is also octanoylated. Thus, octanoylation of ghrelin has been conserved in vertebrates over millions of years of evolution (reviewed in Kojima and Kangawa, 2005 and Sato et al., 2012). Likewise, the GHS-R1a receptor for octanoylated ghrelin has also been highly conserved in vertebrates as far back as zebrafish (Yang et al., 2008).

Mammalian 1 * 10 20 28
Human GSSFLSPEHQRVQQRKESKKPPAKLQPR
Rhesus Monkey GSSFLSPEHQRAQQRKESKKPPAKLQPR
Mouse GSSFLSPEHQKAQQRKESKKPPAKLQPR
Monglian Gerbil GSSFLSPEHQKTQQRKESKKPPAKLQPR
Rat GSSFLSPEHQKAQQRKESKKPPAKLQPR
Dog GSSFLSPEHQKLQQRKESKKPPAKLQPR
Porcine GSSFLSPEHQKVQQRKESKKPAAKLKPR
Sheep GSSFLSPEHQKLQ-RKEPKKPSGRLKPR
Bovine GSSFLSPEHQKLQ-RKEAKKPSGRLKPR

Avian 1 * 10 20 26
Chicken GSSFLSPTYKNIQQQKDTRKPTARLH
Duck GSSFLSPEFKKIQQQNDPTKTTAKIH
Emu GSSFLSPDYKKIQQRKDPRKPTTKLH
Goose GSSFLSPEFKKIQQQNDPAKATAKIH
Turkey GSSFLSPAYKNIQQQKDTRKPTARLHPR

Fish 1 * 10 20 23
Rainbow Trout 1 GSSFLSPSQKPQ**VRQ**GKGK-PPRV-amide
Rainbow Trout 2 GSSFLSPSQKPQGKGK-PPRV-amide
Japanese Eel GSSFLSPSQRPQGKDKKPPRV-amide
Goldfish GTSFLSPAQKPQ--GRRPPRM-amide
Zebrafish GTSFLSPTQKPQ--GRRPPRV-amide
Tilapia GSSFLSPSQKPQNKVK-SSRI-amide

Amphibian 1 * 10 20 27
Bullfrog GLTFLSPADMQKIAERQSQNKLRHGNMN

Figure 4. Sequence comparison of vertebrate ghrelin. Identical amino acids in each species of mammal, bird, and fish are colored. The asterisks indicate acyl-modified third amino acids. NH2-terminal cores with acyl-modification sites are well conserved among all vertebrate ghrelins. [Reproduced from (Kojima and Kangawa, 2005)].

Octanoylation of the Ser^3 is the most unique post-translational modification of ghrelin. Ghrelin is the only protein currently known to be octanoylated. The enzyme that catalyzes the octanoylation of ghrelin was identified by two individual studies and designated as ghrelin O-acyltransferase (GOAT) in 2008 (Gutierrez et al., 2008; Yang et al., 2008). GOAT is a member of the family membrane-bound O-acyltransferases (MBOAT), with its structure conserved among different species. It was proposed that pro-ghrelin is octanoylated before it is transported to the Golgi where it is cleaved by PC1/3 to form mature ghrelin, in agreement with Zhu and co-workers (Zhu et al., 2006), suggesting that GOAT is located in ER (Yang et al., 2008). Transcripts for both GOAT and ghrelin are present predominantly in stomach and pancreas (Gutierrez et al., 2008). Genetic disruption of the GOAT gene in mice leads to complete absence of octanoylated ghrelin in circulation (Gutierrez et al., 2008). *In vitro* analysis demonstrated that GOAT activity could be significantly inhibited by an octanoylated ghrelin pentapeptide and other end-products (Yang et al., 2008), suggesting the existence of a negative feedback regulation. In addition to the octanoylation, different acylation by other fatty acids has been reported, this subject will be explored further is this chapter (section: "*Other ghrelin forms*").

C-ghrelin and obestatin

Recent evidence suggests that the 66 amino acid tail of pro-ghrelin (52–117) (Figure 3) can either circulate as a full-length peptide (C-ghrelin) or be processed to smaller peptides, mainly obestatin. Pemberton and co-workers demonstrated the presence of C-ghrelin in human plasma that circulates at higher concentrations than ghrelin, and is elevated in heart failure (Pemberton et al., 2003), suggesting that it may be bioactive (reviewed in Soares and Leite-Moreira, 2008).

Obestatin, a 23-amino acid peptide derived from polypeptide C-ghrelin of pro-ghrelin, was proposed as a novel endogenous ligand for the orphan GPCR Gpr39 (Zhang et al., 2005). This new peptide was named *obestatin* based on its appetite-suppressing potential, thus this discovery brought exciting new insights to the gut peptide field. It was found that intraperitoneal or intracerebroventricular injection of obestatin in mice inhibited the food intake (Zhang et al., 2005). In addition, it was reported that peripheral injection of obestatin inhibited jejunal contraction, suppressed gastric emptying and decreased body-weight gain (Zhang et al., 2005). However, these findings could not be reproduced by several groups (Gourcerol et al., 2006; Holst et al., 2007; Yamamoto et al., 2007), and must therefore be interpreted with caution (reviewed in Garg, 2007).

Other ghrelin forms

In humans, multiple ghrelin-derived molecules differing on the amino acid length and the type of acylation at Ser^3 have also been isolated from the stomach and plasma (Hosoda et al., 2003) (Figure 5). Although the major active form of human ghrelin is a 28-amino acid peptide with an octanoyl modification (C8:O) at Ser^3, the ghrelin-derived molecules observed include octanoyl ghrelin-(1–27), decanoyl (C10:O) ghrelin, decanoyl ghrelin-(1–27) and decenoyl (C10:1) ghrelin (Hosoda et al., 2003). When the C-terminal arginine residue (Arg^{28}) is removed from ghrelin, most likely by a carboxypeptidase B-like enzyme, ghrelin-(1–27), with 27 amino acids, is produced (Hosoda et al., 2003). In human stomach, the processing product ratio of the 27-amino acid to the 28-amino acid peptide was observed to be ~1:3 (Hosoda et al., 2003). It is likely that 27- and 28-amino acid ghrelin molecules are produced through alternative C-terminal processing of the same pro-ghrelin precursor. All of the acyl-modified ghrelins and acyl-modified ghrelin-derived molecules have showed the same potency to induce an increase of intracellular Ca^{2+} concentration in the GHS-R1a-expressing cells and stimulate GH release in anesthetized rats (Hosoda et al., 2003).

Figure 5. Various ghrelin variants resulting from alternative splicing (des-Gln14-ghrelin) or post-translational modification. Biologically active analogues of ghrelin and ghrelin-(1–27) (missing the last amino acid arginine at position 28) with acyl chains of 8, 10 or 11 C atoms or without the acyl post-translational modification were described. [Adapted from (Korbonits et al., 2004)].

Moreover, des-acyl ghrelin and des-acyl ghrelin-(1–27), lacking the acyl post-translational modification, were found in the human stomach as well as in the plasma (Hosoda et al., 2003). Ghrelin is the predominant form in stomach tissue (Hu et al., 2005), however the des-acyl ghrelin was found in circulation even at far greater concentrations than ghrelin, with the ratio of ghrelin to des-acyl ghrelin in plasma being 0.25 (Hosoda et al., 2000a) or 0.1 (Gauna et al., 2007) in rodents and 0.2 in humans (Marzullo et al., 2004), suggesting that when newly synthesized and mature ghrelin is secreted, it is rapidly deacylated in circulation (this and other aspects related with ghrelin degradation are reviewed in Satou et al., 2011). Des-acyl ghrelin, however, does not bind do GHS-R1a (Hosoda et al., 2000a), does not displace ghrelin to rat hypothalamus or pituitary membranes, and is unable to stimulate GH release *in vivo* (Hosoda et al., 2000a; Torsello et al., 2002), being considered initially an inactive ghrelin-derived peptide. However, a conspicuous number of recent reports strongly suggest that des-acyl ghrelin has intrinsic activities in a variety of physiological and pathophysiological situations (reviewed in Delhanty et al., 2012), breaking a paradigm from 1999 (Kojima et al., 1999).

Finally, GOAT might also acylate ghrelin with other fatty acids, besides octanoate, ranging from acetate to tetradecanoic acid (Gutierrez et al., 2008). Peak intensities for acyl-modified forms of ghrelin corresponding to C7 to C12 appear most intense (Gutierrez et al., 2008), thus suggesting that GOAT is also responsible for the acylation of these various ghrelin variants.

Minimal sequence of ghrelin for receptor activation

The first 4 or 5 residues of ghrelin (Gly–Ser–Ser(n-octanoyl)–Phe–Leu) are sufficient for calcium mobilization *in vitro* (Bednarek et al., 2000). The acylation of the hydroxyl group of Ser^3 with longer aliphatic chains or with unsaturated or branched octanoyl groups does not affect the activity of ghrelin (Bednarek et al., 2000), while shorter acetyl groups, less hydrophobic, dramatically decreased the activity of the peptide. When the n-octanoyl group was transferred to the Ser^2 instead of Ser^3 the biological activity of this peptide was similar to that of the natural ghrelin (Bednarek et al., 2000). Hence, the N-terminal [Gly–Ser–Ser(n-octanoyl)–Phe] segment constitutes the "essential core" required for efficient binding to and activation of GHS-R1a (reviewed in Korbonits et al., 2004).

Tissue Distribution of Ghrelin

Stomach and other gastrointestinal organs

In all vertebrate species, the stomach is the primary organ producing ghrelin (Ariyasu et al., 2001). Ghrelin is present in X/A-like cells, which account for about 20% of the endocrine cell population in adult oxyntic glands (Date et al., 2000a). The granule contents of X/A-like cells had not been identified until the discovery of ghrelin. The X/A-like cells contain round, compact, electron dense granules that are filled with ghrelin (Figure 6) (Date et al., 2000a; Dornonville de la Cour et al., 2001; Yabuki et al., 2004). The gastric X/A-like cells can be stained with an antibody that is specific to the NH2-terminal, acyl-modified portion of ghrelin, indicating that ghrelin in the secretory granules of X/A-like cells has already been acyl-modified. Indeed, the concentration of ghrelin found in the circulation of rats decreases by 80% following surgical removal of the acid-producing part of the stomach suggesting that the oxyntic mucosa is the major source of ghrelin (Dornonville de la Cour et al., 2001). A similar drop of plasma ghrelin levels was found in humans following gastrectomy (Leonetti et al., 2003).

Ghrelin-immunoreactive cells are also found in the duodenum, jejunum, ileum, and colon (Date et al., 2000a; Hosoda et al., 2000a; Sakata et al., 2002), among which ghrelin concentration gradually decreases from the duodenum to the colon. As in the stomach, the main molecular forms of intestinal ghrelin are *n*-octanoyl ghrelin and des-acyl ghrelin (Date et al., 2000a). The pancreas is another ghrelin-producing organ [ghrelin and des-acyl ghrelin both exist in pancreas (Date et al., 2002b)], especially during the embryo development and the production of ghrelin decreases rapidly after birth (Chanoine and Wong, 2004). However, the cell type that produces ghrelin in the pancreatic islets remains controversial. In contrast, gastric ghrelin levels are low during the prenatal period and increase after birth (Hayashida et al., 2002).

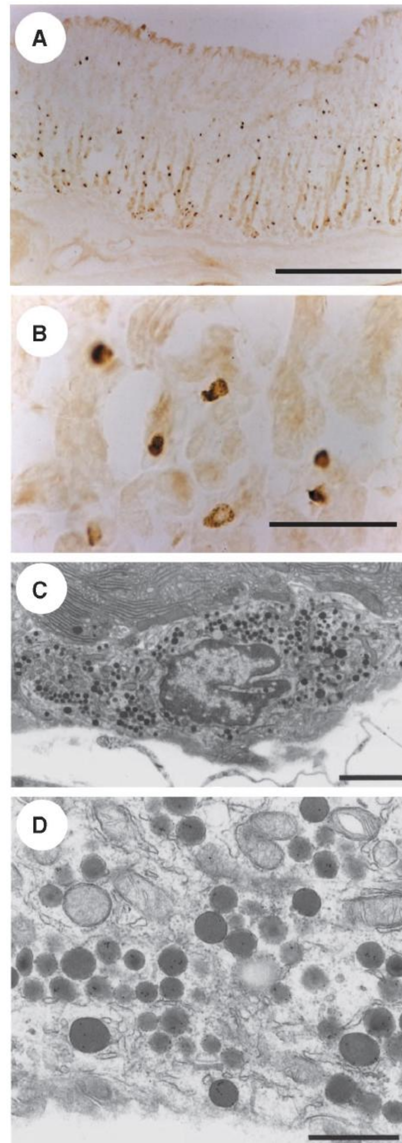


Figure 6. Ghrelin cells in the stomach. (**A**) Ghrelin-immunoreactive cells in the stomach are found from the neck to the base of the oxyntic gland. Scale bar, 400 μm. This distribution pattern is typical for gastric endocrine cells. (**B**) High magnification of A. Scale bar, 40 μm. (**C**–**D**) Representative immunoelectron photographs of a ghrelin-producing cell in the oxyntic gland. (**C**) This ovoid cell has many round, compact, electron-dense granules in its cytoplasm. Scale bar, 2 μm. (**D**) High magnification of C. Scale bar, 500 nm. Granules in the cytoplasm are labeled with immunogold staining for ghrelin. [Reproduced from (Kojima and Kangawa, 2005)].

Central nervous system

Since the ghrelin receptor (or GHS-R1a) is mainly expressed in the hypothalamus and pituitary (Howard et al., 1996; Guan et al., 1997), its endogenous ligand has been thought to exist mainly in the hypothalamic regions. Despite that, the content of ghrelin in the brain is very low (Kojima et al., 1999; Hosoda et al., 2000a).

Ghrelin has been found in the hypothalamic arcuate nucleus (Kojima et al., 1999; Lu et al., 2002), an important region for controlling appetite. In addition, ghrelin immunoreactivity was detected in a previously uncharacterized group of hypothalamic neurons in the internuclear

space between the lateral hypothalamus (LH), the arcuate nucleus (ARC), the ventromedial (VMH), the dorsomedial (DMH), and the paraventricular (PVH) hypothalamic nuclei and the ependymal layer of the third ventricle (Cowley et al., 2003). Ghrelin was present in the axon terminals and these axons innervated the ARC, VMH, PVH, DMH, and the LH, as well as outside the hypothalamus to the bed nucleus of the stria terminalis, amygdala, thalamus, and habenula (Cowley et al., 2003). These histological findings are consistent with the functional studies in which injection of ghrelin into the cerebral ventricles of rats potently stimulates food intake (Tschöp et al., 2000; Nakazato et al., 2001).

Ghrelin *in vivo* administration has a strong GH-releasing activity (Kojima et al., 1999; Arvat et al., 2000, 2001; Date et al., 2000b; Peino et al., 2000; Takaya et al., 2000). This finding, together with the expression of ghrelin receptor in the pituitary (Howard et al., 1996; Guan et al., 1997), suggest that the GHS-R1a is expressed in pituitary cells. Additionally, ghrelin has also been found in the pituitary gland where it may influence the release of GH in an autocrine or paracrine manner (Korbonits et al., 2001a, 2001b).

Other tissues

Two major forms of ghrelin: *n*-octanoyl and des-acyl ghrelin, are found in circulation (Hosoda et al., 2000a). Moreover the ghrelin peptide has been shown to be expressed in the lung (Volante et al., 2002), immune cells (Hattori et al., 2001), placenta (Gualillo et al., 2001), cyclical expression in the ovary (Caminos et al., 2003; Gaytan et al., 2003), testis (Barreiro et al., 2002; Tena-Sempere et al., 2002), and kidney (Mori et al., 2000).

Ghrelin transport across the blood brain-barrier

The blood-brain barrier (BBB) prevents the unrestricted exchange of substances between the central nervous system and the blood and also conveys information between the central nervous system and the gastrointestinal tract through several mechanisms.

Human ghrelin is readily transported by a saturable system across BBB in both directions, whereas the transport of mouse ghrelin is saturable in only the brain-to-blood direction (Banks et al., 2002). Mouse ghrelin differs from human ghrelin in two of their 28 residues, with lysine replacing arginine at position 11 and alanine replacing valine at position 12. These two amino acids are, therefore, critical for recognition by the blood-to-brain transporter but not the CNS-to-blood transporter (Banks et al., 2002). In addition, mouse ghrelin could be occasionally demonstrated to cross the BBB, although uptake is not clearly detectable (Banks et al., 2002) (Figure 7).

The mouse des-acyl ghrelin enters the brain by nonsaturable transmembrane diffusion and is sequestered once within the central nervous system, whereas mouse obestatin does not

cross the blood-brain barrier (Banks et al., 2002) (Figure 7). Small amounts of internalized obestatin show rapid intracellular degradation (Pan et al., 2006). The extent and direction in which ghrelin can cross the BBB is therefore influenced by at least two features of its primary structure: i) the post-translationally added fatty acid, and ii) the amino acid sequence.

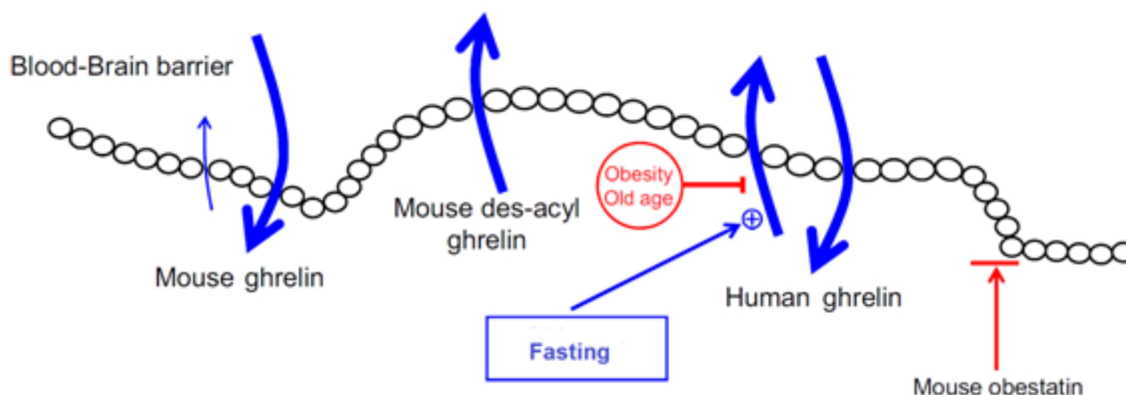


Figure 7. Differential transportation of mouse ghrelin, mouse des-acyl ghrelin, human ghrelin, and mouse obestatin across the blood-brain barrier, which regulates communications between the gut and the brain. Obesity, old age, and fasting affect ghrelin across the blood-brain barrier. [Adapted from (Chen et al., 2009)].

Moreover, physiological status may affect the transport of ghrelin gene products across the blood-brain barrier (Figure 7). First, obesity and old age decrease the transport of intravenous human ghrelin across the blood-brain barrier, resulting in an inverse relationship between body weight and ghrelin blood-brain barrier permeability (Banks et al., 2008). Second, fasting tends to enhance ghrelin transport across the blood-brain barrier (Banks et al., 2008). Collectively, starvation would augment orexigenic effects of ghrelin on the central nervous system by increasing its crossing of the blood-brain barrier, whereas obesity and old age would attenuate the actions of ghrelin on the brain by decreasing its crossing through the blood-brain barrier.

Peripherally injected ghrelin stimulates hypothalamic neurons (Hewson and Dickson, 2000; Rüter et al., 2003) and stimulates food intake (Wren et al., 2001a; Date et al., 2002a). However, emerging evidence reveals that the orexigenic effects by peripheral ghrelin could occur via indirect mechanisms and be dependent on the vagal afferent pathway (Asakawa et al., 2001a; Date et al., 2002a; le Roux et al., 2005; Gilg and Lutz, 2006), whereas GH release induced by peripheral ghrelin could occur via direct mechanism (le Roux et al., 2005). In agreement with this, the ghrelin receptor was detected in vagal afferent neurons in the rat nodose ganglion suggesting that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve (Sakata et al., 2003; Zhang et al., 2004; reviewed in Kojima and Kangawa, 2005).

To determine whether human ghrelin can enter in the brain, Diano and co-workers analyzed the presence of radiolabed ghrelin in various parts of the mouse brain. The olfactory

bulb and occipital cortex had the highest uptake, followed by the hypothalamus, while the hippocampus had the lowest uptake, similar to the whole brain (Diano et al., 2006). Moreover, unlabeled human ghrelin inhibited the uptake into the hypothalamus and hippocampus, suggesting a specific saturable uptake (Diano et al., 2006). In conclusion, although peripheral ghrelin appears to cross the blood-brain barrier entering directly into the brain, peripheral (vagal) connections to the brainstem also could play an important role in mediating the effect of ghrelin; thus, the extent of ghrelin transport from the periphery to the brain needs further studies.

Regulation of Ghrelin Release

The regulation of ghrelin and consequently its effects could occur at several levels: i) transcription and translation of the ghrelin gene; ii) addition of post-translational modification; iii) secretion rate of ghrelin from cells in the stomach, hypothalamus and other sites; iv) binding proteins in the circulation; v) deactivation of circulating ghrelin; vi) transport across the blood–brain barrier; vii) clearance of ghrelin via the kidney or liver; viii) influence of other ligands of the ghrelin receptor(s); ix) the expression of the ghrelin receptor(s), x) and intracellular signaling of ghrelin receptor(s) (reviewed in Yin et al., 2009).

Fasting and food intake

The most important factor for the regulation of ghrelin secretion is feeding. Endogenous ghrelin levels change according to acute as well as chronic nutritional status. Fasting causes elevation of ghrelin levels. Plasma ghrelin levels increase nearly two-fold immediately before each meal, and fall to trough levels within 60–120 min after food intake (Tschöp et al., 2000, 2001a; Ariyasu et al., 2001; Cummings et al., 2001, 2002b) (Figure 8).

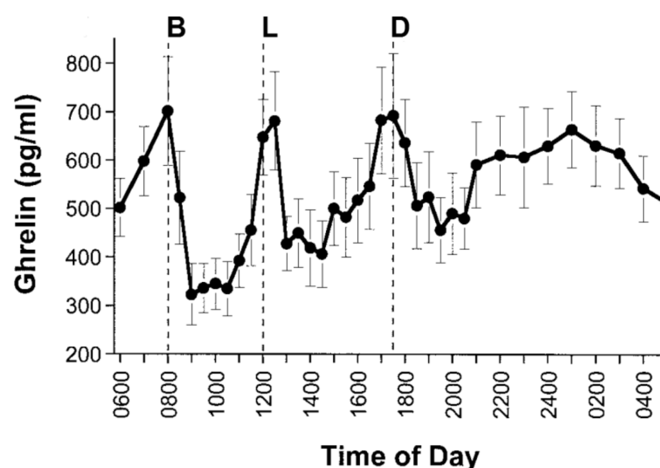


Figure 8. Average plasma ghrelin concentrations during a 24-h period in 10 human subjects consuming breakfast (B), lunch (L), and dinner (D) at the times indicated (8.00h, 12.00h, and 17.30h, respectively). [Adapted from (Cummings et al., 2001)].

It is still not clear which factors are involved in the regulation of ghrelin secretion upon fasting and after food intake (postprandial) (reviewed in Patterson et al., 2011). The postprandial ghrelin suppression is proportional to the ingested calorie load (Callahan et al., 2004). The increased ghrelin during fasting is a result of increased pulse frequency as well as pulse amplitude, which together with the synchronized lower leptin (orexigenic compound) pulses result in a powerful orexigenic effect (Bagnasco et al., 2002). Fasting increases stomach ghrelin expression, but not pituitary or hypothalamic ghrelin expression (Torsello et al., 2003). Additionally, prolonged fasting for 3 days did not change ghrelin levels significantly compared to the baseline state suggesting that the meal-related changes are rather decreases after food intake than increases due to fasting (Chan et al., 2004). Moreover, it was found that caloric restriction in mice with a diet containing 60% of normal calories for ten days (60% caloric restriction) resulted in a four-fold increase in circulating levels of ghrelin (Lutter et al., 2008).

Ghrelin levels change throughout the day with especially high levels before food intake and during the night, and a reduction immediately after food intake (Figure 8) suggesting that ghrelin might be an important factor in meal initiation [(Ariyasu et al., 2001; Cummings et al., 2001; Tschöp et al., 2001a; Shiiya et al., 2002; Faulconbridge et al., 2003), although this has not been seen in all studies (Barkan et al., 2003)]. The preprandial increase of ghrelin levels correlates well with hunger scores in humans initiating meals voluntarily, without time- or food-related cues, further supporting the possible role for ghrelin in meal initiation (Cummings et al., 2004).

Obesity-related diseases and stress

Plasma ghrelin concentration is low in obese people and high in lean people (Tschöp et al., 2001b; Bellone et al., 2002; Cummings et al., 2002b; Hansen et al., 2002; Shiiya et al., 2002; Haqq et al., 2003; Rosická et al., 2003) (Figure 9). Consistently, plasma ghrelin level is highly increased in anorexia nervosa patients and returns to normal levels upon weight gain and recovery from the disease (Otto et al., 2001; Cuntz et al., 2002; Tanaka et al., 2003b; Soriano-Guillén et al., 2004). Ghrelin concentration is also increased in bulimia nervosa patients (Tanaka et al., 2003a). Moreover, chronic social defeat stress (CSDS) in mice increases significantly the levels of ghrelin that persist for at least 4 weeks (Lutter et al., 2008), in agreement with studies that have demonstrated increases in gastric ghrelin mRNA or total plasma ghrelin after acute stress (Asakawa et al., 2001b; Kristenssson et al., 2006).

Patients after gastric bypass lose their weight, and their circulating ghrelin levels decrease (Cummings et al., 2002b; Geloneze et al., 2003; Leonetti et al., 2003) (Figure 9). Moreover, plasma ghrelin levels in subjects who underwent gastric bypass did not oscillate in relation to meals and were markedly lower than those of both lean controls and matched obese controls, altogether confirming that the stomach is the main site of ghrelin production (Cummings et al., 2002b) (Figure 9). Finally, plasma ghrelin concentration also decreases in

patients with short bowel syndrome (Krsek et al., 2003), probably due to the loss of ghrelin-producing tissues.

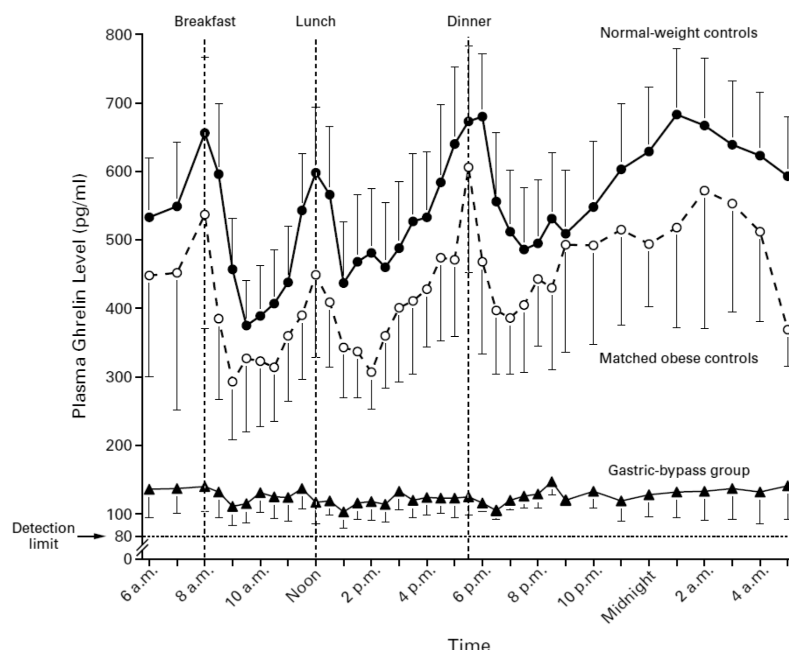


Figure 9. 24-hour plasma ghrelin profiles in subjects who underwent gastric bypass and in controls. The study groups represented are 5 obese subjects who underwent a proximal Roux-en-Y gastric bypass, 10 normal-weight controls, and 5 obese subjects who had recently lost weight by dieting and were matched to the subjects in the gastric-bypass group according to final body-mass index, age, and sex. Breakfast, lunch, and dinner were provided at the times indicated. [Reproduced from (Cummings et al., 2002b)].

Ghrelin Receptor

The human ghrelin receptor or growth hormone secretagogue receptor (GHS-R) gene, similarly to the ghrelin gene, is located on chromosome 3, at position 3q26.2, and encodes for two transcripts, the transcript 1a which encodes a full-length receptor (GHS-R1a) and the transcript 1b which codifies for a shortened version (GHS-R1b) (McKee et al., 1997a). The 1a type encodes a typical 7 seven transmembrane domains (TMs) G-coupled protein receptor (GPCR) with binding and functional properties consistent with its role as the ghrelin receptor (reviewed in Davenport et al., 2005).

The ghrelin receptor gene

The GHS-R gene (Figure 10) consists of three exons; the first codifying exon (exon 1) encodes TM-1 to TM-5, and the second codifying exon (exon 2) encodes TM-6 to TM-7 (McKee et al., 1997a). Type 1b is derived from only the exon 1 and encodes only five of the seven predicted TM domains. The type 1b receptor is thus a COOH-terminal truncated form of the type 1a receptor (McKee et al., 1997a). A 5'-untranslated first exon (exon 0) was also described, characteristic of other G protein-coupled receptors (Kaji et al., 1998).

GHSR-1b does not have, until now, known biological activity, and fails to bind GHSs (Howard et al., 1996; reviewed in Smith et al., 1999, van der Lely et al., 2004 and Davenport et al., 2005). However, it has been proposed that this truncated ghrelin receptor may modulate the function of full-length version of the receptor. Indeed, GHS-R1a and GHS-R1b can exist as heterodimers when heterologously expressed in HEK 293 cells, and the truncated version of the receptor appears to act as a dominant negative of the full-length GHS-R1a, decreasing its cell surface expression and consequently its constitutive activity (Leung et al., 2007). Recently, it was demonstrated that GHS-R1a is retained in the endoplasmic reticulum by heterodimerization with GHS-R1b, attenuating its constitutive activity due to the decrease of cell surface expression of GHS-R1a (Chow et al., 2012), in agreement with the previous finding.

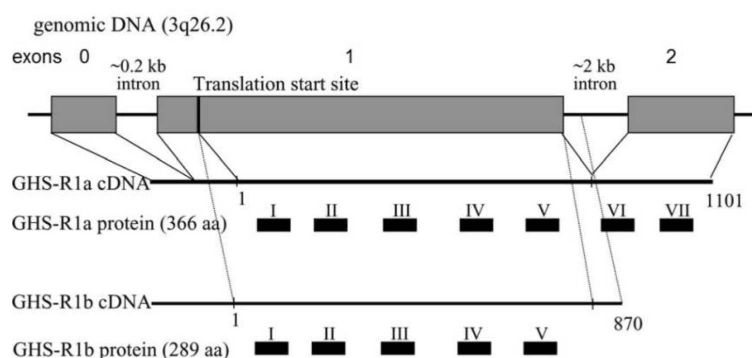


Figure 10. GHS-R gene. Gray boxes represent exons. Black boxes represent the transmembrane domain marked with roman numbers. The last 24 amino acids of the GHS-R1b are transcribed from the intronic sequence. [Adapted from (Korbonits et al., 2004)].

GHS-R1a structure

The human GHS-R1a is a polypeptide of 366 amino acids with a molecular mass of approximately 41 kDa (Howard et al., 1996; reviewed in Petersenn, 2002 and Camiña, 2006) and belongs to family A of GPCRs (reviewed in Bockaert and Pin, 1999). GPCRs span the membrane with seven α-helix hydrophobic domains forming the receptor core, joint by three alternate intra- and extracellular domains, beginning with an extracellular N-terminal domain and ending with an intracellular C-terminal domain (reviewed in Bockaert and Pin, 1999). The GHS-R1a has features characteristic of a typical GPCR, including conserved cysteine residues in the first two extracellular loops, several potential sites for post-translational modifications (*N*-linked glycosylation and phosphorylation), and an aromatic triplet sequence (E/DRY) located immediately after TM-3 in the second intracellular loop (reviewed in Bockaert and Pin, 1999 and Petersenn, 2002).

Based on its deduced peptide sequence, GHS-R1a is not obviously related to known subfamilies of GPCRs, although it is often included in a small family of receptors for small polypeptides comprising the receptor for motilin (52% homology), neurotensin receptor-1 (NTS-R1) and NTS-R2 subtype (33–35% homology), neuromedin U receptor-1 (NMU-R1) and NMU-

R2 subtype (~30% homology), and the orphan receptor Gpr39 (27–32% homology) (McKee et al., 1997b; Tan et al., 1998), named GHS-R1a homologues. The ghrelin receptor is well conserved across all vertebrate species examined, including a number of mammals, chicken, and pufferfish (Smith et al., 1999, 2001; Palyha et al., 2000). This strict conservation suggests that ghrelin and its receptor serve important physiological functions.

Remarkable differences in the binding profile among ghrelin, synthetic peptidyl (hexarelin) and non-peptidyl (MK-0677) GHSs have been reported (Ong et al., 1998a, 1998b; Muccioli et al., 2000; Papotti et al., 2000), mostly in tissues that do not express GHS-R1a or express the receptor at a low level, suggesting the presence of a novel unidentified receptor (reviewed in Muccioli et al., 2007). For instance, the heart possesses GHS-binding sites specific for peptidyl GHSs only (Bodart et al., 1999; Muccioli et al., 2000; Papotti et al., 2000). Additionally, the existence of various GHS-R1a homologues, and the splice variant GHS-R1b, the lack of a definite phenotype in GHS-R1a knockout mice (growth, development, appetite and body composition), as well as the presence of multiple endogenous ghrelin-like ligands, suggest that other receptors for ghrelin and GHSs may exist.

Finally, the evidence for the existence of heteromeric complexes containing GHS-R1a and others GPCRs (D_1, D2, MC_3, 5-HT_{2C} and Gpr83) (Jiang et al., 2006; Rediger et al., 2009, 2011; Kern et al., 2012; Müller et al., 2013; Schellekens et al., 2013) increases the complexity of ghrelin receptor-mediated signaling, and must be taken into account when considering the ghrelin receptor as a therapeutical target (reviewed in Mear et al., 2013).

Distribution

The ghrelin receptor is ubiquitously distributed in the brain. GHS-R1a mRNA expression was detected in multiple hypothalamic nuclei (Figure 11) and in the pituitary gland (Howard et al., 1996; Guan et al., 1997; Zigman et al., 2006). This distribution is consistent with its physiological function associated with energy metabolism and GH-release. In addition, GHS-R1a is also expressed in the cerebral cortex (piriform cortex), and parts of the midbrain [e.g., ventral tegmental area (VTA) and substancia nigra)], pons, medulla oblongata and hippocampus (Figure 11) (Howard et al., 1996; Guan et al., 1997; Zigman et al., 2006).

This pattern of expression has been confirmed using binding assays using labelled ghrelin; in particular, binding of ghrelin was observed in the hypothalamus, hippocampus and cortex (Cowley et al., 2003; Diano et al., 2006; Harrold et al., 2008), as well as in the olfactory bulb (Diano et al., 2006) and VTA (Abizaid et al., 2006). Of particular interest, binding was predominantly localized to presynaptic boutons in the arcuate nucleus (ARC), lateral hypothalamus (LH), paraventricular nucleus of hypothalamus (PVH), and cortex (Cowley et al., 2003), suggesting a presynaptic localization of ghrelin receptor in these regions.

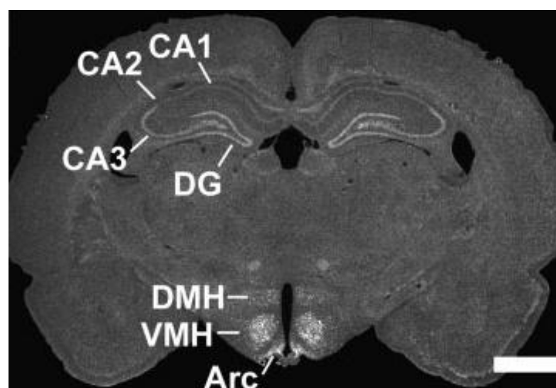


Figure 11. Low-power photomicrograph summarizing GHS-R type 1a mRNA expression sites in the hippocampus and hypothalamus, in rat brain. Scale bar, 2 mm. Hypothalamic dorsomedial (DMH), ventromedial (VMH), arcuate (ARC) nuclei and dentate gyrus (DG). [Reproduced from (Zigman et al., 2006)].

Regarding the hippocampus, GHS-R1a mRNA expression has been observed in CA1, CA2 and CA3 subfields of Ammons's horn, as well as in the dentate gyrus (Guan et al., 1997; Zigman et al., 2006) (Figure 11). At the protein level, this pattern of expression was confirmed when assessed by immunohistochemistry (Diano et al., 2006; Cuellar and Isokawa, 2011; Berrout and Isokawa, 2012; Shi et al., 2013). In particular, the ghrelin receptor was detected both in the cell soma and dentrites in the CA1 subfield, and exhibited a punctate pattern of labeling along the dendritic shaft (Berrout and Isokawa, 2012). However, in this study, the authors were not able to determine whether these puncta colocalized with dendritic spines (Berrout and Isokawa, 2012), although they suggest a postsynaptic localization of the ghrelin receptor in this hippocampal region.

Moreover, it was found that mRNA expression in rats is undetectable in the fetal hippocampus, but detectable at high levels at postnatal day 7 (P7) (Katayama et al., 2000), suggesting that ghrelin receptor expression is regulated during development in the hippocampus. Moreover, Lattuada and co-workers found that GHS-R1a mRNA expression increases up to 16 days *in vitro* (DIV) in primary rat hippocampal and cortical neurons, decreasing subsequently at later stages in culture (21 DIV) (Lattuada et al., 2013). Altogether, these observations suggest a role for ghrelin in hippocampal-dependent processes. Indeed, this assumption was confirmed, reviewed in the section: "*Cognitive enhancing effect of ghrelin and ghrelin receptor agonists".*

In the peripheral tissues GHS-R1a mRNA expression was demonstrated in many organs, including heart, lung, liver, kidney, pancreas, stomach, small and large intestines, adipose tissue, thyroid gland, adrenal gland, spleen, and immune cells in rodents and humans (Guan et al., 1997; Hattori et al., 2001; Kojima et al., 2001; Gnanapavan et al., 2002). Such a wide distribution of GHS-R1a is consistent with the reported functions beyond the control of GH release and food intake (reviewed in Chen et al., 2009).

Signaling pathways

The binding of ghrelin to GHS-R1a causes conformational changes of intracellular loops of the receptors, which expose binding sites to G proteins. The coupling of G proteins to GHS-R1a promotes guanosine diphosphate (GDP) release and guanosine triphosphate (GTP) binding to the G protein α subunit, thus activating G protein subunits to initiate intracellular signaling cascades by acting on various downstream effectors. Proposed signal transduction mechanisms underlying ghrelin function involve the regulation of ionic currents and protein phosphorylation-based intracellular signaling (reviewed in Gao and Horvath, 2007 and Muccioli et al., 2007).

Once bound to ghrelin, activated GHS-R1a normally binds the $G_{q/11}\alpha$ subunit of a G protein, which leads to activation of phosphatidylinositol-specific phospholipase C (PI-PLC) to generate inositol 1,4,5-triphosphate (IP_3) and diacylglicerol (DAG) from phosphatidylinositol 4,5-diphosphate (PIP_2) (Howard et al., 1996; Smith et al., 1997). The intracellular free calcium (Ca^{2+}) concentration increases because of the rapid, though transient, release of Ca^{2+} from IP_3-responsive cytoplasmatic storage pools in the endoplasmic reticulum (ER). Increased Ca^{2+} and DAG levels activate protein kinase C (PKC), which, in turn, inhibits the potassium (K^+) channels, causing depolarization (reviewed in Camiña, 2006). This depolarization causes the opening of voltage-dependent L-type Ca^{2+} channels, causing more sustained accumulation of intracellular Ca^{2+}. Together with the blockade of K^+ channels, the intracellular rise in free Ca^{2+} exacerbates the cellular depolarization (Figure 12).

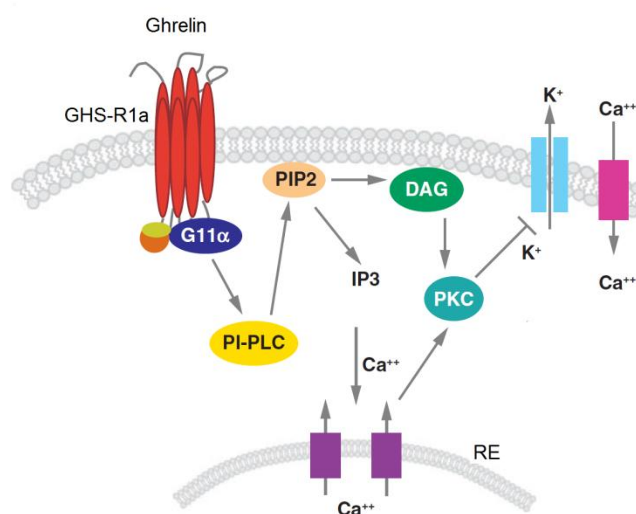


Figure 12. Prototypical signaling of GHS-R1a. Ligand binding to the GHS-R1a activates phospholipase C (PI-PLC) to hydrolyze phosphatidylinositol 4,5-diphosphate (PIP_2), stored in the plasma membrane, to give both diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). A rapid transient elevation of calcium (Ca^{2+}) levels is a result of Ca^{2+} release from intracellular stores (endoplamic reticulum, ER) in response to IP_3, which together with DAG activates protein kinase C (PKC) in the plasma membrane. PKC inhibits the potassium (K^+) channel, causing depolarization: this depolarization causes the opening of voltage-dependent L-type Ca^{2+} channels. [Adapted from (Gao and Horvath, 2007)].

Ghrelin also activates MAP kinase (MAPK) and PI3 kinase (PI3K) cascades in different cellular systems to promote proliferation (Kim et al., 2004a, 2004b; Mazzocchi et al., 2004), through various G protein subunits including $G_q\alpha$ and a pertussis toxin (PTX)-sensitive G protein ($G_{i/o}\alpha$) (reviewed in Camiña, 2006). Moreover, ghrelin induces an increase in intracellular Ca^{2+} concentration through N-type Ca^{2+} channels influx in isolated neuropeptide Y producing hypothalamic neurons (Kohno et al., 2003). Because N-type Ca^{2+} channels are modulated by cAMP-dependent protein kinase A (PKA) activation, this suggest that GHS-R1a is coupled to $G_s\alpha$ in NPY-producing neurons in the hypothalamus (Kohno et al., 2003).

Additionally, in porcine somatotropes, ghrelin-stimulated GH secretion depends on activation of nitric oxide/cyclic guanosine monophosphate (cGMP) signaling (Rodríguez-Pacheco et al., 2005). Again in the hypothalamus, it was found that ghrelin enhances the activity of 5'-AMP-activated protein kinase (AMPK) (Andersson et al., 2004), regulating the energy balance (reviewed in Carling, 2005). However, the molecular mechanism by which ghrelin regulates AMPK is still unknown. Ghrelin can also inhibit vascular inflammation through the activation of the calmodulin-dependent kinase kinase (CaMKK), AMPK and endothelial nitric oxide synthase (eNOS) (Xu et al., 2008). Other signaling pathways coupled to GHS-R1a are Raf-MEK-MAPK and PI3K/Akt/GSK3β (reviewed in Frago et al., 2011). Altogether, these observations suggest that GHS-R1a generates intracellular signaling mainly through the $G_{q/11}\alpha$ subunit of a G protein; however the specific intracellular pathways elicited by this receptor seem to be dependent on the tissue type in which it is expressed.

Constitutive activity

Signal transduction from the extracellular environment via GPCRs in general requires a conformational change from an inactive (R) to an active state (R*). Certain GPCRs are stabilized in an active conformation without any ligand present. The ability to propagate the intracellular signal in the absence of agonist is commonly known as constitutive activity (reviewed in Aloyo et al., 2010). The number of GPCRs displaying constitutive activity with clear physiological implications is not vast, but it is steadily growing (reviewed in Bond and Ijzerman, 2006).

The GHS-R1a exhibits an unusual high constitutive activity, triggering intracellular signaling with ~50% of its maximal capacity in the absence of the agonist, ghrelin (Holst et al., 2003). The molecular basis of such activity appears to be related with three aromatic residues located in TM6 and TM7, namely PheVI:16 (phenylalanine residue at the position 16 in the transmembrane domain 6), PheVII:06 and PheVII:09 (reviewed in Mear et al., 2013). This region promotes the formation of a hydrophobic core between helices 6 and 7, to ensure proper docking of the extracellular end of TM7 into TM6, mimicking agonist activation and stabilizing the receptor in the active conformation (Holst et al., 2004). TM6 and TM7 movements into their inward-bend promoting the ligand-independent active conformation can be stoically blocked

using a modified substance P inverse agonist [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P (Holst et al., 2006).

Because it is well established that GHS-R1a generates intracellular signaling mainly through the $G_{q/11}\alpha$ subunit of a G protein (reviewed in Gao and Horvath, 2007), determination of inositol phosphate (IP) accumulation was used as measure of constitutive activity in COS-7 cells transfected with the human GHS-R1a. The ghrelin receptor displays a high degree of constitutive signaling through the phospholipase C pathway, demonstrated by the gene dose-dependent but ligand-independent increase in IP_3 production, which is approximately 50% of the maximal agonist-induced activity (Holst et al., 2003, 2004).

Holst and co-workers also found, in HEK-293 cells, that the transfection of GHS-R1a resulted in a dose-dependent stimulation of the cAMP-responsive element (CRE) pathway as monitored by a reporter assay using CRE-driven luciferase activity (Holst et al., 2003, 2004). The activity of the transcription factor CRE binding protein (CREB) is generally believed to be controlled by cAMP-dependent kinases, however it can also be activated by downstream kinases of the $G_q\alpha$ pathway, such as Ca^{2+}/calmodulin kinase IV or PKC (Matthews et al., 1994; Singh et al., 2001). In spite of the well established involvement of the $G_q\alpha$ type of G proteins in the ghrelin receptor-mediated signaling, it is impossible to discard the involvement of $G_s\alpha$ and cAMP in the agonist-independent signaling, because there are some indications that GHS-R1a is also coupled to this subunit of G proteins (Kohno et al., 2003; Cuellar and Isokawa, 2011).

Performing serum responsive element (SRE) reporter assay on HEK-293 cells transfected with GHS-R1a, Holst and co-workers observed a 10-fold increase in the ligand-independent signaling compared to the cells transfected with the empty plasmid (Holst et al., 2004). This increase was partially blocked by the Rho kinase inhibitor Y-27632, suggesting the involvement of the G protein system $G_{13}\alpha$, which is often functionally coupled to Rho signaling pathway (Ponimaskin et al., 2002). Finally, GHS-R1a does not show any constitutive activity in the pituitary cell line RC-4B/C.40, indicating that GHS-R1a constitutive activity may depend on the cellular context (Falls et al., 2006). While many other signaling pathways have been shown to play a role in the GHS-R1a ligand-dependent activation, further investigation is needed to better understand which signaling pathways are involved in the GHS-R1a ligand-independent activation. In particular, it is still unknown whether the GHS-R1a ligand-independent CRE-activation passes through the calcium/calmodulin kinase and/or via the cAMP/PKA pathways (Figure 13).

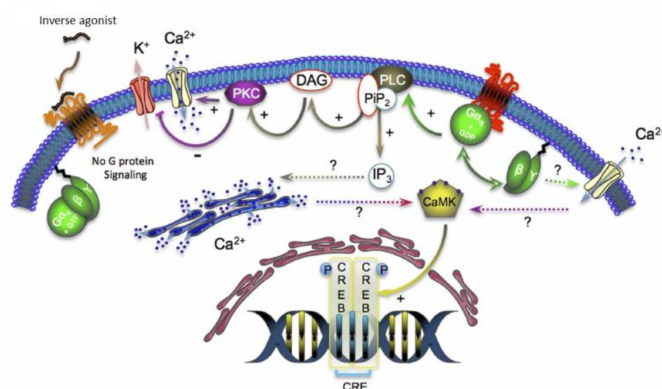


Figure 13. GHS-R1a constitutive activity leads to the dissociation of α and βγ subunits of heterotrimeric G proteins. The free $G_q\alpha$ subunit activates the PLC that cleaves PIP_2 into IP_3 and DAG. DAG activates the PKC, which in turn actives the Ca^{2+} channels and inhibits the K^+ channels (continuous line).The effects of IP_3 on the intracellular Ca^{2+} mobilization and the origin of the Ca^{2+} that activates the Ca^{2+} calmodulin/kinase (CaMK) remain unclear and will need further investigations (dotted line). It is not even clear whether the $G_s\alpha$-mediated signaling is activated or not. The binding of the inverse agonist to the GHS-R1a inhibits the G protein signaling and decreases the IP_3 (via PLC) and CRE pathway (via phosphorylated CREB) constitutive activation. [Adapted from (Mear et al., 2013)].

Recently, it has been suggested that the co-expression of the GHS-R1a receptor with its truncated splicing variant 1b decreases its constitutive activity. GHS-R1a receptors can be retained in the ER by heterodimerization with GHS-R1b, decreasing the constitutive activation of phospholipase C due to the decrease in the cell surface expression of GHS-R1a (Leung et al., 2007; Chow et al., 2012).

Several reports have now demonstrated the physiological relevance of GHS-R1a constitutive activity. Wang and co-workers identified two 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), associated with obesity and short stature, respectively (Wang et al., 2004). Both amino acids are highly conserved among humans, swine, and rats (Wang et al., 2004). A recent study has re-stated that an Ala204Glu missense allele segregates with both short stature and obesity (Pantel et al., 2006).

Both mutated residues have been implicated in the constitutive signaling of GHS-R1a. Phe279 (PheVII:06) is part of an unusual cluster of aromatic residues located on the inner faces of TM VI and VII, which are essential in holding the ghrelin receptor in the active conformation. Removal of the aromaticity at position 279 (VII:06) selectively eliminates the constitutive activity (Holst et al., 2004). When GHS-R1a, carrying the mutation Ala204Glu, is transfected in HEK-293 cells, the expressed receptor displays reduced constitutive activity and lower expression at the plasma membrane (Pantel et al., 2006). However, ghrelin bound with normal affinity and stimulated signal transduction with normal potency and normal apparent efficacy (Pantel et al.,

2006). Altogether, these observations suggest that selective loss of ghrelin receptor constitutive activity causes a syndrome of short stature and obesity (reviewed in Holst and Schwartz, 2006).

Recently the constitutive activity of the ghrelin receptor was implicated in the control of food intake and body weight *in vivo*. The intracerebroventricular injection of the inverse agonist of the ghrelin receptor [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P significantly decreased the food intake and body weight in rats (Petersen et al., 2009). In addition, 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). The most surprising finding of this study is that an inverse agonist, which does not affect the ghrelin-induced food intake, was able to inhibit the food intake and prevent body weight gain observed in the control groups. Recently, it was also found that GHS-R1a constitutive activity increases limbic seizures in rodents (Portelli et al., 2012). Although the recent evidence indicating that ghrelin receptor constitutive activity plays a role in various physiological functions, most of the studies performed so far focused on the molecular aspects. Thus, certainly many questions are still pending in this field, in particular regarding the physiological relevance of this unusual and uncharacterized aspect of ghrelin receptor. The answer to these questions may lead to developing novel and unique therapies for various disorders (reviewed in Mear et al., 2013).

Physiological Functions of Ghrelin

Both ghrelin and its receptor are ubiquitously expressed in the periphery as well as in the central nervous system suggesting the involvement of ghrelin-mediated signaling in a plethora of biological functions (Table I) (reviewed extensively in Korbonits et al., 2004, van der Lely et al., 2004, Kojima and Kangawa, 2005, 2010, Gao and Horvath, 2007, Chen et al., 2009 and Sato et al., 2012). Among the physiological functions of ghrelin, here we will focus on its very well described role on the regulation of appetite. Additionally we will also discuss ghrelin's function on obesity, anxiety and food reward.

Table I. Physiological functions of ghrelin in humans or rats. ACTH, adrenocorticotrophic hormone; AMPK, 5'-AMP-activated protein kinase; GH, growth hormone; PRL, prolactin. [Adapted from (Sato et al., 2012)].

Functions	Effects	Organs	Species
Pituitary hormone secretions			
GH	↑	pituitary	humans, rats
PRL	↑ (weak)	pituitary	humans
ACTH	↑ (weak)	pituitary	humans
Appetite regulations			
Food intake	↑		humans, rats
AMPK activity	↑	hypothalamus	rats
Lipid metabolisms			
Adiposity	↑		rats
Triglyceride	↑	white adipose tissue, liver	rats
Glucose metabolisms			
Blood glucose	↑		humans
Insulin	↓	Pancreas	humans
Cardiovascular functions			
Blood pressure	↓		humans, rats
Cardiac output	↑		rats
Gastric functions			
Gastric acid secretion	↑	Stomach	rats
Gastric movement	↑	Stomach	rats
Bone metabolism			
Osteoblast differenciation	↑	Bone	rats
Bone mineral density	↑	Bone	rats

↑, stimulate; ↓, decrease.

Appetite regulation

Feeding is a basic behavior that is necessary for life. Long-term lack of food results in death. It is well accepted that appetite is controlled by the brain and that feeding behavior is regulated by complex mechanisms in the central nervous system, in particular the hypothalamus. For instance, removal of the lateral hypothalamus causes hypophagia (decreased feeding), leading to death due to severe weight loss, while removal of the ventromedial hypothalamus causes hyperphagia (increased feeding) (reviewed in Schwartz et al., 2000, Vettor et al., 2002 and Druce and Bloom, 2003). Thus feeding is regulated by a balance of stimulating and inhibiting forces in the hypothalamus. Recent identification of appetite-regulating humoral factors reveals regulatory mechanisms not only in the central nervous system but also mediated by factors secreted from peripheral tissues (reviewed in Neary et al., 2004, Small and Bloom, 2004, Ukkola, 2004 and Wynne et al., 2004). Leptin, produced in adipose tissues, is an appetite-suppressing (anorexigenic) factor that transmits satiety signals to the brain (reviewed in Friedman, 2002). Hunger (orexigenic) signals from peripheral tissues, remained unidentified until the discovery of ghrelin (Kojima et al., 1999).

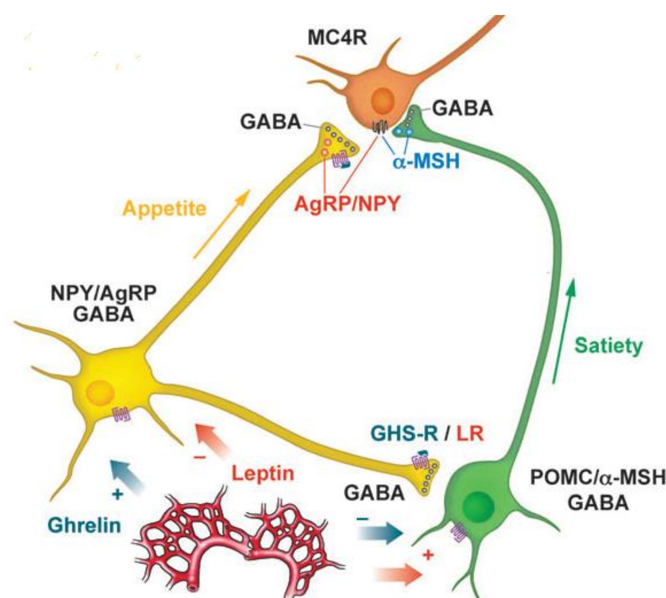


Figure 14. Two subsets of neurons in the ARC have opposite effects on feeding. Activation of POMC cells by leptin promotes satiety triggered by the release of POMC-driven α-MSH at target sites where it binds to melanocortin 4 receptor (MC4R). NPY neurons also express agouti-related peptide (AgRP). These cells are inhibited by leptin but stimulated by ghrelin, which promotes feeding, decreases energy expenditure, and silences POMC neuron firing. The effect of the NPY cells is mediated by three distinct mechanisms: i) GABA, ii) NPY, and iii) AgRP, an inverse agonist of MC4R antagonizing the anorexigenic effects of α-MSH. [Adapted from (Gao and Horvath, 2007)].

Among the hypothalamic regions involved in the appetite regulation, the arcuate nucleus (ARC) has a pivot role (Figure 14). This region contains two subsets of neurons that have opposite effects on feeding, and which are located in an anatomically strategic place, because they are close to fenestrated capillaries at the base of the hypothalamus, giving them access to various humoral signals that are restricted from other portions of the brain (Benoit et al., 2000; Cone et al., 2001). One set of neurons expresses pro-opiomelanocortin (PMOC) and when stimulated they produce anorexigenic effects (Boston et al., 1997). The POMC precursor is cleaved into melanocyte-stimulating hormones (α-, β-, and γ-MSH), β-endorphin, and adrenocorticotrophic hormone (ACTH) (Cone, 2005). Of these, α- and β-MSH reduce food intake and body weight and increase energy expenditure in animals and humans (Fan et al., 1997; Biebermann et al., 2006; Lee et al., 2006b). Both α- and β-MSH act on melanocortin receptor subtypes 3 and 4 (MC3/4R) (Adan et al., 1994), which are abundant particularly in the ARC (Mountjoy et al., 1994). The second group of cells in which increased activity leads to orexigenic responses contain neuropeptide Y (NPY) and the agouti gene-related peptide (AgRP) (Ollmann et al., 1997; Baskin et al., 1999). NPY potently stimulates feeding and reduces energy expenditure (Clark et al., 1984; Stanley and Leibowitz, 1984). It is found throughout the central nervous system (Sahu et al., 1988), with its highest concentration in the ARC (Chronwall et al., 1984). AgRP is also orexigenic. It acts as a natural antagonist of MC3/4R (Ollmann et al., 1997), thereby reducing the anorexigenic effect of α-MSH. Ghrelin from the plasma increases the rate of secretion of NPY and AgRP. Moreover, it stimulates presynaptic GHS-R located in

the NPY cells axons connecting to POMC cells, inducing the release of the inhibitory neurotransmitter GABA to inhibit the POMC neurons. On the other hand, plasma leptin triggers the release of α-MSH and inhibits the release of NPY and AgRP. Thus, ghrelin and leptin have a competitive interaction in feeding regulation (appetite *vs* satiety) (Figure 14).

Moreover, it has been suggested that ghrelin-expressing neurons in the hypothalamus also have a important role on regulation of appetite (Cowley et al., 2003). In the ARC, these ghrelin-containing neurons send efferent fibers onto NPY- and AgRP-expressing neurons to stimulate the release of these orexigenic peptides; and also stimulate the release of GABA from these neurons, which acts in the POMC-containing neurons, suppressing the release of anorexigenic peptides (Cowley et al., 2003). Ghrelin-expressing neurons also send efferent fibers onto NPY neurons in the PVH; however, in this hypothalamic nucleus the neuronal network of ghrelin-expressing neurons is more complex (Cowley et al., 2003; reviewed in Korbonits et al., 2004).

Ghrelin on the onset of appetite and obesity

Ghrelin is currently the only described orexigenic hormone from the periphery, which acts centrally to modulate the energy homeostasis. The important role of ghrelin in the regulation of appetite and satiety is well established and the orexigenic effects of peripheral or central ghrelin administration are widely documented in rodents (Tschöp et al., 2000; Wren et al., 2000, 2001b; Asakawa et al., 2003; Finger et al., 2011a, 2011b; Rolland et al., 2011), as well as in (lean and obese) humans (Wren et al., 2001a; Druce et al., 2005). Plasma ghrelin levels rise prior to food ingestion and under conditions of caloric restriction and decrease postprandially, and considerable evidence supports a role for ghrelin in the sensation of hunger and meal initiation, increasing food intake and body weight (Cummings et al., 2001; Tschöp et al., 2001a, 2001b; Lutter et al., 2008). In rodents, ghrelin-induced gain weight is based on accretion of fat mass gain by reducing fat utilization (Tschöp et al., 2000).

In addition, ghrelin appears relevant for some types of human obesity (reviewed in Hillman et al., 2011). The marked and prolonged weight loss induced by Roux-en-Y gastric bypass (RYGB) surgery is thought by many to be enhanced by post-bypass reductions in circulating ghrelin. As first reported in 2002, 24-hour ghrelin profiles of RYGB subjects were >70% lower than those of obese control subjects (Cummings et al., 2002b). Most subsequent RYGB trials have confirmed this relative ghrelin deficiency, as opposed to the rise in ghrelin observed with dieting or other instances of energy insufficiency (reviewed in Cummings et al., 2005, Lee et al., 2006a and Thaler and Cummings, 2009). While most obese individuals have reduced baseline levels of circulating ghrelin as compared with normal subjects (reviewed in Hillman et al., 2011), in Prader-Willi Syndrome elevated ghrelin levels exist and have been postulated by some authors to contribute to the unrelenting hyperphagia and weight gain characteristic of this syndromic form of obesity (Cummings et al., 2002a; Tauber et al., 2004).

These findings have supported the notion that blocking ghrelin action may be an effective strategy to reduce body weight or prevent the development of obesity (Zorrilla et al., 2006). In fact, reduction of bioavailable ghrelin or daily administration of GHS-R1a antagonists to diet-induced obese mice lower body weights and reduce food intake (Shearman et al., 2006; Zorrilla et al., 2006; Esler et al., 2007; Rudolph et al., 2007).

Ghrelin and anxiety

Both central and peripheral administration of ghrelin induce anxiogenesis, when assessed by the elevated plus maze test (Asakawa et al., 2001b; Carlini et al., 2002, 2004; Carvajal et al., 2009). In agreement with this idea, anti-sense oligonucleotides against ghrelin produced an anxiolytic response (Kanehisa et al., 2006). These findings support the notion that ghrelin promotes anxiogenesis.

It was found that rodents and/or humans with cachexia of various etiologies and anorexia nervosa have high circulating ghrelin (Otto et al., 2001; Wisse et al., 2001; Tolle et al., 2003; Garcia et al., 2008). It was hypothesized that the endogenous ghrelin elevations associated with cachexia and anorexia nervosa serve as a protective function against what otherwise would be a more severe phenotype (reviewed in Perelló and Zigman, 2012 and Schellekens et al., 2012). In agreement, Lutter and co-workers found that the enhanced ghrelin plasma levels induced by stress help to minimize depressive-like symptoms of chronic stress (Lutter et al., 2008), suggesting that ghrelin can also produce anxyolitic and anti-depressive responses. In agreement with this idea, Spencer and co-workers found that in the absence of endogenous ghrelin, mice are more anxious after acute stress, and exogenous ghrelin reversed this effect (Spencer et al., 2012), suggesting a physiological relevance of endogenous ghrelin in stress and anxiety. The reasons for the conflicting functions of ghrelin on stress (anxiety vs. anxiolytic function) are currently unclear (reviewed in Andrews, 2011). It was also suggested that the genetic ablation of ghrelin receptor increases the anxiety-like behavior when assessed by open field test in mice (Albarran-Zeckler et al., 2012), suggesting additionally a role as anxiolytic for ghrelin mediated-signaling. The implication of ghrelin in mood is further supported by the discovery of polymorphisms in the gene encoding ghrelin that are associated with depression (Nakashima et al., 2008), and the evidence that ghrelin levels can be decreased in depressed patients (Barim et al., 2009).

Food reward behavior

The sensation of appetite triggers signaling pathways to initiate food intake when nutrients are low. However, food also elicits pleasurable and rewarding signals, mediated via dopamine release in the mesolimbic circuitry system, which can override satiety and stimulate appetite independently of metabolic needs (reviewed in Kenny, 2011). The non-homeostatic motivational factors to obtain a food reward increase with food palatability and caloric content

and the resulting over-consumption is being recognised as a key component in the underlying causes for the increase in obesity incidence (reviewed in Schellekens et al., 2012).

The non-homeostatic feeding involves extra-hypothalamic neurocircuitry, including cortical areas, as well as areas within the mesolimbic dopaminergic pathway, including the ventral tegmental area (VTA), nucleus accumbens, hippocampus and amygdala (reviewed in Skibicka and Dickson, 2011). Additionally, the extensive glutamatergic afferents to dopaminergic neurons from regions involved with sensory (insula or primary gustatory cortex), homeostasis (hypothalamus), reward (nucleus accumbens), emotional (amygdala and hippocampus), and multimodal (orbifrontal cortex for salience attribution) processes, modulate their activity in response to rewards and to conditioned cues (reviewed in Geisler and Wise, 2008 and Volkow et al., 2011).

Ghrelin has recently emerged as one of the major contributing factors to reward-driven feeding that can override the state of satiation (reviewed in Diz-Chaves, 2011, Perelló and Zigman, 2012 and Schellekens et al., 2012). Firstly, high levels of the GHS-R1a have been reported in the VTA (Guan et al., 1997; Zigman et al., 2006), while the direct ghrelin administration in the VTA induced overflow of dopamine within the nucleus accumbens as measured by microdialysis in freely moving mice (Jerlhag et al., 2007), which supports a role for ghrelin in the VTA mediated reward signaling.

Microinjection of ghrelin in VTA has been shown to stimulate food intake (Naleid et al., 2005; Abizaid et al., 2006), especially the intake of palatable food (Egecioglu et al., 2010). Moreover, the effects of peripheral ghrelin on food intake were blocked by intra-VTA administration of a GHS-R1a antagonist (Abizaid et al., 2006). Consistent with this, peripheral treatment with a GHS-R1a antagonist decreased preference for palatable food, suppressed the ability of sweet treats to condition a place preference (Egecioglu et al., 2010), and suppressed motivated behavior for rewarding foods, both sweet (Egecioglu et al., 2011; Hansson et al., 2011; Skibicka et al., 2012) and high-fat foods (Perello et al., 2010).

Ghrelin’s actions on food reward also are relevant in humans. In particular, ghrelin administration to human subjects during functional magnetic resonance imaging increases the neural response to food pictures in several brain regions implicated in hedonic feeding, including the amygdala, orbitofrontal cortex, hippocampus, striatum, and VTA (Malik et al., 2008). Altogether, these data support an idea for a physiological role of ghrelin increasing the incentive motivation for natural rewards such as food.

Cognitive Enhancing Effect of Ghrelin and Ghrelin Receptor Agonists

The first report showing that ghrelin could affect cognition was by Carlini and co-workers, who demonstrated that ghrelin increases in a dose-dependent manner the latency time in the step-down test (inhibitory avoidance) after intracerebroventricularly (ICV) injection in rats, when given immediately post training, suggesting an increase in memory retention (Carlini et al., 2002). In order to more precisely define the exact site of ghrelin action, the same group then repeated the experiment and used intraparenchymal injections of increasing ghrelin concentrations in the hippocampus, amygdala and dorsal raphe nucleus (DRN) (Carlini et al., 2004). Following behavioral testing they concluded that ghrelin provides a dose-dependent increase in memory, with the maximal effect occurring in the hippocampus (Carlini et al., 2004). Consistent with this result, ghrelin (infusion into the dorsal hippocampus of both hemispheres) significantly improved spatial memory in rats, assessed with the Morris water maze test (Chen et al., 2011). Furthermore, it was found that intra-hippocampal ghrelin injection improves memory retention when administered previous to the training session in the step-down test, but not when administered previous to the test session, suggesting that ghrelin could modulate specific molecular intermediates involved in memory acquisition and/or consolidation, but not in memory retrieval (Carlini et al., 2010a). Diano and co-workers observed that a single subcutaneous injection with ghrelin in rats, before testing the animals, produced a marked improvement of alternation performance in a spontaneous alternation plus-maze task, suggesting an enhancement on spatial memory (Diano et al., 2006). They further analyzed the influence of ghrelin ICV administration, immediately after training, on retention performance in T-maze foot-shock avoidance and step-down passive avoidance tasks in mice (Diano et al., 2006). Ghrelin, in a dose-dependent manner, improved retention in both tasks when administrated immediately after training, suggesting an effect on memory consolidation (Diano et al., 2006) in agreement with the results obtained by Carlini and co-workers (Carlini et al., 2010a). Additionally, using SAMP8 mice (with a phenotype mimicking pathological and cognitive signs of Alzheimer disease), ghrelin improved retention of T-maze foot shock avoidance in 12-month old and 14-month old mice (Diano et al., 2006). Interestingly, ghrelin knockout mice did not exhibit increased exploration time of the novel object, suggesting impairment in their memory of the previous objects (Diano et al., 2006). However, this functional deficiency disappeared rapidly upon ghrelin subcutaneous administration, suggesting that endogenous ghrelin has a physiological role on improving learning and memory performance.

The effect of treatment with ghrelin receptor agonists in hippocampal-dependent memory processes was also evaluated. The subcutaneous injection of the ghrelin mimetic LY444711 produced a marked improvement of alternation performance in a spontaneous alternation plus-maze task (Diano et al., 2006). The oral administration (GSG894490A) and the subcutaneous administration (CP-464709-18) of two structurally-related non-peptide ghrelin

receptor agonists significantly improved performance in the novel object recognition and modified Morris water maze tests (Atcha et al., 2009). Ghrelin receptor knockout mice also display impairments in Morris water maze test (Davis et al., 2011). In disagreement, Albarran-Zeckler and co-workers found that ghrelin receptor knockout mice have improved spatial memory in the Morris water maze test (Albarran-Zeckler et al., 2012). One explanation for these discrepancies between studies might be the age. Studies that compared 8-12 months old to 3 month old wild-type C57BL/6 mice have shown deficits in older mice in Morris water maze test (Gower and Lamberty, 1993).

Finally, ICV administration of ghrelin in neonatal chicks, after training, induced an impairment of memory performance in the passive avoidance task, suggesting a conserved evolutionary role for ghrelin in memory regulation in vertebrates (Carvajal et al., 2009). In agreement, ICV injection of ghrelin, which stimulates feeding in rats, strongly suppressed feeding in neonatal chicks (Furuse et al., 2001; Saito et al., 2005). Why ghrelin suppresses rather than stimulating food intake and memory retention in neonatal chicks remains to be elucidated. Such discrepancies may be due to species properties or the affected site in the brain. These results indicate that species properties are a relevant factor in the ghrelin-mediated effects. Altogether, these results suggest that ghrelin receptor mediated-signaling is an important modulator of hippocampal-dependent cognition. Specifically, ghrelin is thought to play a role in memory retention.

The ability to locate food sources, remember those locations, and recall whether all available food has been consumed are evolutionarily important skills for survival (Moran and Gao, 2006). Energy would not be wasted looking in low-probability sites or returning to sites where all the food had already been eaten (Moran and Gao, 2006), thus suggesting the necessity of a tight connection between feeding-associated behavior and energy balance. Ghrelin may control higher brain functions (in addition to its role as a meal initiation factor) and represent this molecular link between learning capabilities associated to feeding behavior and energy metabolism. However, the precise molecular mechanisms that underlie the effects of ghrelin on hippocampal function remain to be elucidated. In particular, the effect of ghrelin receptor-mediated signaling on long-term potentiation (LTP). The LTP-related mechanism, which is considered the molecular and cellular model for activity-dependent synaptic plasticity in the hippocampus (Bliss and Collingridge, 1993), and a substrate for spatial memory and memory retention (Pavlides et al., 1991; Böhme et al., 1993), will be explored later in this chapter (section "*The glutamatergic synapse*"). In that section the excitatory synapse will be introduced, as well as the glutamate receptors [in particular, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)- and (NMDA) *N*-methyl-D-aspartate (NMDA)-type receptors], which are required for the induction as well as for the expression of this form of plasticity (reviewed in Collingridge et al., 2004).

Cellular and Molecular Mechanisms Underlying the Cognitive Enhancing Effect of Ghrelin Receptor-Mediated Signaling

The spine density hypothesis

Diano and co-workers showed that peripherally administered (intraperitoneal injection) ghrelin crosses the blood-brain barrier, accumulates and binds to neurons in the hippocampus, increasing dendritic spine formation (Diano et al., 2006). Spine synapse density in the CA1 subfield of the hippocampal formation was significantly higher in ghrelin-treated animals compared to vehicle-treated controls, while in ghrelin knockout mice (*Ghrl*$^{-/-}$) there was a reduction in spine density (Diano et al., 2006). The peripheral injection of ghrelin resulted in a shift in spine density of *Ghrl*$^{-/-}$ animals towards the wild-type values, suggesting that endogenous ghrelin controls spine synapse density (Diano et al., 2006). Additionally it was recently shown that application of ghrelin for 60 min or 23 h, in organotypic hippocampal slices, increases the phalloidin (mushroom toxin that has a high affinity to polymerized F-actin) fluorescence signal in the stratum radiatum of the CA1 subfield, suggesting an increase in dentritic spine density in this hippocampal region (Berrout and Isokawa, 2012) in agreement with Diano and co-workers (Diano et al., 2006). This increase was blocked in the presence of the GHS-R1a antagonist [D-Lys^3]-GHRP-6 and when ghrelin was removed from the culture medium after 1 h, and the slices incubated for the subsequent 22 h without ghrelin (Berrout and Isokawa, 2012). This result suggests that maintenance of ghrelin-induced increase in spine density may require continuous activation of the ghrelin receptor and downstream signaling mechanisms. Finally, the density of ghrelin receptor did not change after activation, suggesting that the ghrelin receptor may not be internalized by activation (Berrout and Isokawa, 2012).

The LTP hypothesis

To determine whether ghrelin may affect synaptic plasticity in the CA1 subfield, Diano and co-workers treated acute hippocampal slices for 30 min with ghrelin and then performed 10-Hz (200 pulses) stimulation or series of four theta-burst stimuli (TBS). They did not observe effects of ghrelin on LTP induced by TBS, but observed an increase in the excitatory postsynaptic potential (EPSP) slope after 10-Hz stimulation in slices treated with ghrelin (Diano et al., 2006). However, this 10 Hz stimulation is not established as an electrical stimulation protocol for LTP-related hippocampal synaptic plasticity (reviewed in Albensi et al., 2007).

In another study, ghrelin infusion prevented the decline of high frequency stimulation (HFS)-induced LTP *in vivo* (180 min after LTP induction by HFS), in the dentate gyrus (DG), in a MAP kinase-dependent manner (Chen et al., 2011). However, the effect of ghrelin was only

detected on the late-phase of LTP, which is critically dependent on new protein synthesis, and not on the early phase of LTP that lasts for up to 2 h and requires modification of pre-existing proteins (reviewed in Kelleher et al., 2004 and Lynch, 2004). Finally, Carlini and co-workers demonstrated, in acute hippocampal slices, that ghrelin administration into the CA1 subfield of the hippocampus reduces the threshold to generate LTP (using a protocol based in HFS) in the DG, which was blocked by pre-administration of an nitric oxide synthase (NOS) inhibitor (Carlini et al., 2010b).

The serotonin hypothesis

It has also been suggested that the administration of selective serotonin reuptake inhibitors (SSRI), before ghrelin injection (intra-hippocampal), plays an inhibitory effect upon the increase in memory retention elicited by ghrelin (Carlini et al., 2007). Thus, low serotonin levels possibly represent a prerequisite for the expression of the ghrelin effect on memory retention proposed by Carlini and co-workers (Carlini et al., 2002, 2004). In agreement with this idea, the infusion of ghrelin in the CA1 subfield of hippocampus inhibited serotonin release from hippocampal slices, prepared from animals previously trained in the step down test, and which showed to have an increase in latency time when subjected to this behavioral test (Ghersi et al., 2011). This finding, along with the increase in NOS activity observed in rats upon intra-hippocampal injection with ghrelin (Carlini et al., 2010b), led the authors to propose the following possible mechanism: the binding of ghrelin to its receptors in the hippocampus increases intracellular levels of calcium, stimulating NOS activity and leading to an increase of nitric oxide (NO), which inhibits serotonin release (Ghersi et al., 2011). Thus, the increase of NO and decreased levels of serotonin could justify the increase in memory retention upon treatment with ghrelin (Ghersi et al., 2011). It has been suggested that serotonin and SSRI compounds inhibit LTP in various physiological assays (Corradetti et al., 1992; Mori et al. 2001; Kojima et al., 2003; Kim et al., 2006), while NOS inhibitors increase serotonin levels in hippocampus (Wegener et al., 2000). Additionally, they showed that pre-administration of an NOS inhibitor, similarly to SSRI compounds, prevented the effect induced by ghrelin in memory retention when assessed by the step-down test (Carlini et al., 2010b).

The PI3 kinase pathway hypothesis

A single ghrelin infusion induced long lasting potentiation *in vivo* in the DG (reaching a peak 120 min after ghrelin infusion), enhancing both postsynaptic granule cell excitability and presynaptic transmitter release (Chen et al., 2011). Surprisingly, ghrelin-induced potentiation was not attenuated by application of the competitive NMDARs antagonist AP5, suggesting that NMDARs could play minor role in the expression of ghrelin-induced potentiation in the DG (Chen et al., 2011). Ghrelin infusion into the hippocampus also time-dependently enhanced the phosphorylation of Ser^{473} in Akt, a critical downstream molecule in the PI3 kinase (PI3K) pathway; treatment with inhibitors of PI3K blocked the effect of ghrelin infusion on the synaptic

response (Chen et al., 2011). This is the first experimental evidence suggesting activation of the PI3K signaling pathway in the hippocampus by ghrelin, similarly to what was observed in other cellular systems (reviewed in Gao and Horvath, 2007). Finally, the enhancement of spatial memory mediated by infusion of ghrelin, assessed by the Morris water maze test, was prevented by pretreatment with an PI3K inhibitor (Chen et al., 2011).

The PKA pathway hypothesis

Cuellar and Isokawa report evidence that ghrelin treatment for 60 min in hippocampal organotypic slices may activate protein kinase A (PKA) (Cuellar and Isokawa, 2011). They found that ghrelin treatment increases, in a dose-dependent manner, the phosphorylation of CREB at Ser^{133}, an effect that was abolished by the GHS-R1a antagonist [D-Lys^{3}]-GHRP-6 and in the presence of an PKA inhibitor (Cuellar and Isokawa, 2011). Additionally, the increase in CREB phosphorylation induced by ghrelin was blocked by AP5 and a specific inhibitor of GluN2B-containing NMDARs, and by the endocannabinoids anandamide and 2 arachidonoylglycerol (2-AG) [agonists of the type 1 cannabinoid receptor (CB1R), a $G_i\alpha$ protein-coupled receptor] (Cuellar and Isokawa, 2011). The intensity of phospho-GluN1 immunofluorescence increased upon ghrelin treatment (in particular in stratum radiatum of CA1 subfield) compared to control slices, and this increase was blocked in the presence of anandamide and 2-AG (Cuellar and Isokawa, 2011). Of particular interest, Cuellar and Isokawa proposed that in the hippocampus ghrelin stimulates cAMP production and therefore activation of PKA, suggesting that in this region the ghrelin receptor may also be coupled to $G_s\alpha$ protein-associated signaling pathway, as already described in other contexts (reviewed in Muccioli et al., 2007). The authors argue that upon ghrelin stimulation there is activation of PKA resulting in the enhancement of NMDARs function by increasing the phosphorylation of GluN1; however, the phosphorylated residues in GluN1 were not identified, and it is not known whether the described ghrelin-mediated increase on GluN1 phosphorylation depends on PKA activation. Nevertheless, this is an interesting study showing that ghrelin increases the phosphorylation of CREB in the hippocampus through the PKA signaling pathway, as well as the phosphorylation of GluN1, which seems to be negatively regulated by endocannabinoids.

The involvement of NMDARs in ghrelin´s mediated effect in the hippocampus was further confirmed. Goshadrou and co-workers found that the ICV injection of ghrelin, immediately after training session, improved the memory impairments induced by intraperitoneal injection of MK-801 (a non-competitive antagonist of NMDARs), when assessed by a passive avoidance task (Goshadrou et al., 2013), thus suggesting that the beneficial effects of ghrelin on cognition are mediated partly via the NMDARs signaling.

In conclusion, ghrelin receptor-mediated signaling in the hippocampus may affect memory retention by regulating the following processes, eventually through the PI3K or PKA pathways:

i) spine synapse density;

ii) LTP;

iii) serotonin release (LTP);

iv) synaptic transmission (postsynaptic excitability and presynaptic transmitter release);

v) NMDARs function.

These aspects are intimately associated with the excitatory synaptic transmission in hippocampus. In accordance with this and with the aim to understand the molecular and cellular mechanisms underlying the cognitive enhancing effect of ghrelin, the excitatory synapse will be introduced in the next section. The synaptic trafficking of AMPA-type receptors of glutamate will be addressed with particular depth, because it is required for the expression of long-term potentiation (LTP) and long-term depression (LTD), long-lasting experience-dependent changes in the efficacy of synaptic transmission, which may constitute the cellular substrates of learning and memory.

Several studies have revealed that learning induces long lasting changes in the synaptic strength of central glutamatergic synapses (Rioult-Pedotti et al., 2000; Dragoi et al., 2003; Gruart et al., 2006; Whitlock et al., 2006). This plasticity is essential for maintaining memory and learning (Pastalkova et al., 2006) and is associated with membrane trafficking and phosphorylation of synaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptors (AMPARs) (Lee et al., 2003; Takahashi et al., 2003). With these thoughts in mind we will begin by introducing the excitatory glutamatergic synapse, and then discuss in detail the mechanisms that regulate the synaptic trafficking of AMPARs.

The Glutamatergic Synapse

All brain processes, such as the ability to learn and remember and those involved in emotions, are possible because of the incredibly complex connectivity between neurons in the brain. The human brain contains at least 100 billion neurons, each with the ability to influence many other cells. Clearly, sophisticated and highly efficient mechanisms are needed to enable communication among this astronomical number of elements. Such communication is made possible by synapses, the functional contacts between neurons. Two categories of synapses – electrical and chemical can be distinguished on the basis of their mechanisms of transmission.

In the case of electrical synapses, current flows through gap junctions, which are specialized membrane channels that connect two cells. In contrast, chemical synapses enable cell-to-cell communication via the secretion of neurotransmitters; these chemical agents released by the presynaptic neuron produce secondary current flow in the postsynaptic neuron by activating specific receptor molecules. The secretion of neurotransmitters is triggered by the influx of Ca^{2+} through voltage gated channels, which gives rise to a transient increase in Ca^{2+} concentration within the presynaptic terminal. The rise in Ca^{2+} concentration causes synaptic vesicles to fuse with the presynaptic plasma membrane and release their contents into the space between the pre- and postsynaptic cell (synaptic cleft). The neurotransmitter concentration in the synaptic cleft remains high for only a very brief period. Neurons communicate with each other primarily through fast chemical synapses; hence from now on we will devote our attention to this type of synapses.

The neurotransmitter binding to postsynaptic receptors produces a postsynaptic conductance change as ion channels are opened (or sometimes closed). This conductance change typically generates an electrical current, the postsynaptic current (PSC), which in turn changes the postsynaptic membrane potential to produce a postsynaptic potential (PSP). According with the effect of presynaptic stimulation on the PSP, chemical synapses can be classified as excitatory or inhibitory. Excitatory synapses induce an excitatory postsynaptic potential (EPSP) that depolarizes the membrane toward the threshold required for activation of an action potential. Conversely, inhibitory synapses induce an inhibitory postsynaptic potential

(IPSP) that hyperpolarizes the membrane away from the threshold potential (reviewed in Häusser et al., 2000). In other words, excitatory synapses increase the likelihood of a postsynaptic action potential occurring, and inhibitory synapses decrease this likelihood.

Excitatory synapses occur mainly on tiny protrusions from the dentritic shaft called dentritic spines. In contrast, inhibitory synapses are formed on the shaft of dendrites, or on cell bodies and axon initial segments. The postsynaptic side of excitatory synapses differs from inhibitory synapses not only in their content of neurotransmitters receptors but also in their morphology and molecular composition and organization (reviewed in Sheng and Kim, 2011).

The Postsynaptic Density of Excitatory Synapses

Excitatory synapses are characterized by a morphological and a functional specialization of the postsynaptic membrane called the postsynaptic density (PSD) (reviewed in Sheng and Kim, 2011) (Figure 15). The PSD was first observed in electron micrographs as a fuzzy electron-dense thickening of the postsynaptic membrane that is opposed to the presynaptic active zone (Gray, 1959; Siekevitz, 1985), and therefore also called “asymmetric synapses”. The PSD contains the glutamate receptors that are activated by the glutamate neurotransmitter from the presynaptic terminal, as well as associated-signaling molecules and structural molecules. A set of abundant scaffold proteins holds together the PSD by binding to the glutamate receptors, other postsynaptic receptors and adhesion molecules, cytoplasmic signaling enzymes, and cytoskeletal elements.

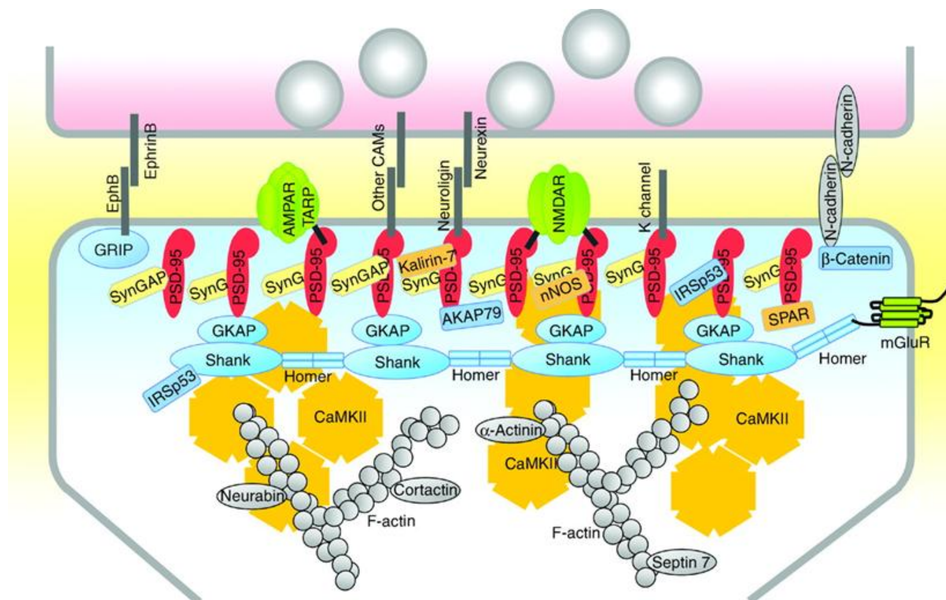


Figure 15. Molecular organization of the PSD of excitatory synapses. Schematic diagram of the major proteins of the PSD, with protein interactions indicated by direct contacts or overlaps between the proteins. [Reproduced from (Sheng and Kim, 2011)].

Glutamate Receptors

The vast majority of excitation in the central nervous system is mediated by glutamate, a ubiquitous amino acid (reviewed in Watkins and Jane, 2006). In neurons, glutamate is packed within synaptic vesicles by dedicated vesicular transporters (vGluTs) and following presynaptic activity it is released in the synaptic cleft. It then diffuses rapidly across the synaptic cleft and activates glutamate receptors localized primarily on the postsynaptic membrane. There are two major classes of glutamate receptors: the ionotropic receptors (iGluRs), ion channels, and the mebotropic receptors (mGluRs), G protein-coupled receptors (GPCRs).

GPCRs are membrane-bound proteins that are activated by extracellular ligands such as light, peptides, and neurotransmitters, and transduce intracellular signals via interactions with G proteins. They mediate slower synaptic responses (occurring over seconds and minutes), rather than milliseconds as occurs for iGluRs. mGluRs are broadly distributed throughout the CNS and are specifically localized at discrete synaptic and extrasynaptic sites in both neurons and glia in virtually every major brain region. Activation of mGluRs results in diverse actions on neuronal excitability and synaptic transmission by modulation of a variety of ion channels and other regulatory and signaling proteins (reviewed in Niswender and Conn, 2010). Because iGluRs are essential for the induction and maintenance of synaptic plasticity, long-term changes in synaptic efficacy that underlie the remarkable capacity of the brain to store and retrieve information over extended periods of time (reviewed in Lüscher and Malenka, 2012), from now on we will focus our attention in this type of receptors.

Ionotropic Glutamate Receptors

Mammalian iGluRs (reviewed in Smart and Paoletti, 2012) are encoded by a total of 18 genes that assemble into four major families the AMPA, kainate, NMDA, and delta receptors, named after their most selective agonist (Watkins et al., 1981) (AMPARs, kainateRs, NMDARs, and deltaRs, respectively) (Hollmann et al., 1989; Seeburg, 1993; Nakanishi and Masu, 1994; Dingledine et al., 1999) (Figure 16).

Ionotropic glutamate receptors (iGluRs)

AMPARs	KainateRs	NMDARs	DeltaRs
GluA1	GluK1 GluK4	GluN1* GluN2A GluN3A*	GluD1
GluA2	GluK2 GluK5	GluN2B GluN3B*	GluD2*
GluA3	GluK3	GluN2C	
GluA4		GluN2D	

* These subunits bind glycine or D-serine.

Figure 16. The four families of iGluRs. Each subunit is encoded by a distinct gene. There is no known mixing between families. [Reproduced from (Smart and Paoletti, 2012)].

KainateRs are found both on presynaptic and postsynaptic compartments and have a more modulatory rather than direct role at synapses (reviewed in Lerma, 2006 and Contractor et al., 2011). DeltaRs are the least understood iGluRs largely because, in contrast to other iGluRs, their apparent incapacity to gate an ion channel following ligand binding makes them electrically "silent" (Kohda et al., 2000; Schmid et al., 2009). The excitatory postsynaptic current (EPSC) is typically mediated by members of the AMPAR and NMDAR families (Figure 17).

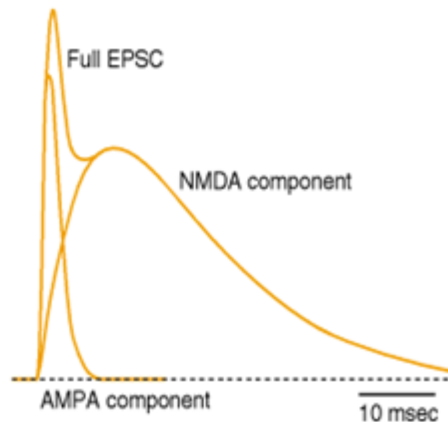


Figure 17. Excitatory postsynaptic currents (EPSCs) comprise both-AMPA- and NMDA-receptor components. Treatment with the NMDA receptor blocker AP5 or the AMPA receptor antagonist NBQX reveals the other component in isolation, as shown. The EPSC is composed of a brief AMPA receptor component and a more prolonged NMDA receptor component. [Reproduced from (Siegel et al., 2006)].

Synaptic release of glutamate results in a two-component excitatory postsynaptic current (EPSC) upon binding to AMPA and NMDA receptors. Activation of AMPA receptors mediates a component that has rapid onset and decay, whereas the component mediated by NMDA receptor activation has a slower rise time and a decay lasting up to several hundred milliseconds (Figure 17). The long time course of NMDA receptor activation provides more opportunity for temporal and spatial summation of multiple inputs.

AMPA and NMDA receptors contain ion channels that conduct fluxes of Na^+, Ca^{2+} and K^+ (Figure 18A). Activation of AMPA receptors leads to strong influx of Na^+ and only a small efflux of K^+, such that the net effect is depolarization of the postsynaptic neuron. Hence, the influx of current through GluA2-containing AMPA receptors (most AMPARs contain at least one subunit of GluA2) is normally largely carried by the movement of Na^+ from the extracellular face to the intracellular compartment; these receptors have very low Ca^{2+} permeability. Following transcription, the GluA2 subunit mRNA undergoes RNA editing, whereby the RNA coding for a glutamine (Q) residue in the channel pore-forming region (within the short reentrant membrane M2 loop) is exchanged for the RNA coding for arginine (R), this site has thus been named "Q/R site" (Sommer et al., 1991). The Q/R site also provides a key endoplasmic reticulum (ER) quality control check point, attenuating the formation of GluA2 homomeric channels (Greger et al., 2002). The large arginine residue located in the pore region of the channels limits the flow of

Na^+ and K^+ ions and prevents divalent ions from entering the cell. Thus, most AMPARs carry inward currents at negative potentials and outward currents at positive potentials, and the reversal potential is 0 mV, i.e., the current–voltage relationship is linear (Figure 18B).

On the other hand, GluA2-lacking AMPA receptors (e.g., GluA1 homomeric channels or GluA1/3 heteromeric channels) have a glutamine in the pore region instead of the arginine residue present in edited GluA2 subunits. Such channels have a high conductance for Na^+ and are permeable to Ca^{2+}. Because endogenous polyamines, which are negatively charged, can also access a site close to the cytoplasmic mouth of the pore, the channels are inhibited at positive potentials. In consequence, GluA2-lacking AMPA receptors have an inward-rectifying current–voltage relationship (i.e., they conduct current more easily into the cell than out of the cell; Figure 18B).

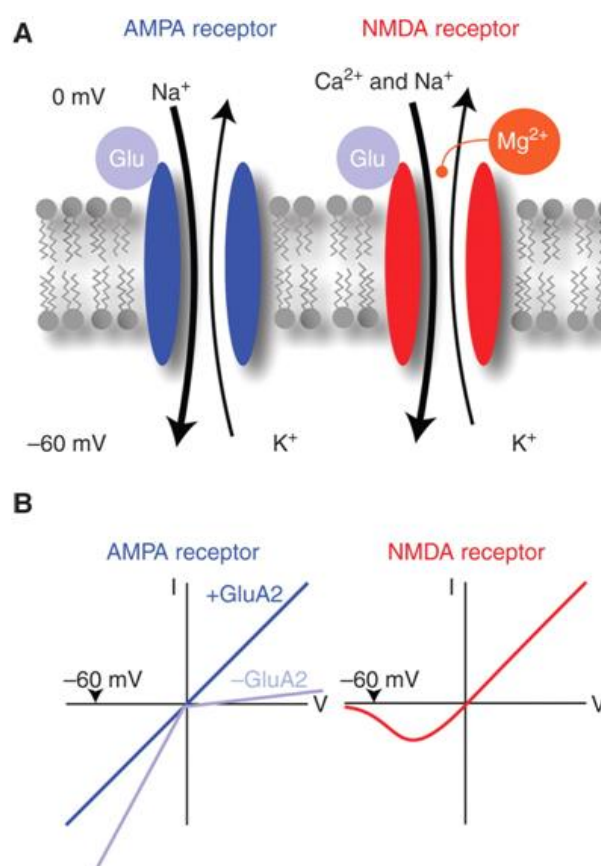


Figure 18. Biophysical properties of synaptic AMPA and NMDA receptors. (**A**) When glutamate binds to AMPA receptors, many sodium ions flow into the cell while only some potassium ions leave the neuron, causing a net depolarization of the membrane. NMDA receptors are also permeable to calcium but only if the magnesium ion is expelled by a slight depolarization of the neuron. (**B**) The current–voltage (*I*–*V*) relationship provides a biophysical signature for the different receptors. AMPA receptors have a linear *I*–*V* relationship when they contain the subunit GluA2, but are inward-rectifying without GluA2. NMDA receptors have a complex *I*–*V* curve because Mg^{2+} blocks the pore at negative potentials. [Reproduced from (Lüscher and Malenka, 2012)].

The current–voltage relationship of NMDARs is more complex. At resting and hyperpolarized membrane potentials the concentration of Mg^{2+} in the extracellular fluid is sufficient to virtually abolish ion flux through NMDA receptor channels even in the presence of glutamate and the coagonist glycine. Thus, although glutamate and glycine are bound to their receptive sites and the channel is “activated”, the entry of Mg^{2+} into the channel pore blocks the movement of ions through the channel. In the presence of Mg^{2+} ions NMDA receptor channels exhibit a characteristic J-shapped current–voltage relationship (Figure 18B). As the membrane potential is made less negative or even positive, the affinity of Mg^{2+} for its binding site decreases and the block becomes ineffective. It is important to note that NMDARs conduct currents only when glutamate is bound and the postsynaptic neuron is depolarized. Hence, pre- and postsynaptic neurons need to be active to open NMDARs. Through this mechanism NMDARs play the role of molecular coincidence detectors, essential for several forms of synaptic plasticity.

AMPA Receptors

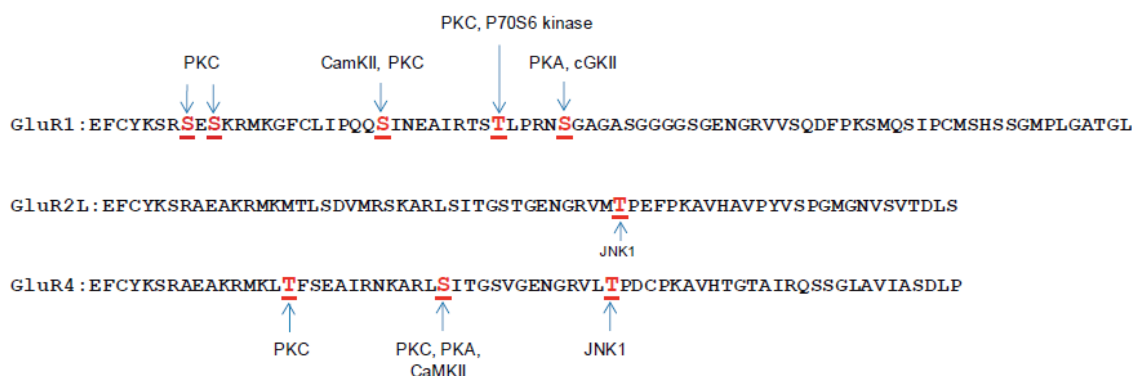
AMPARs are the principal transducers of fast excitatory neurotransmission in the mammalian brain, and are targets for multiple signaling pathways that regulate the strength of glutamatergic synapses. AMPARs are tetrameric assemblies of highly homologous subunits encoded by four different genes, GluA1-4. The trafficking of AMPARs into and out of synapses is highly dynamic and is regulated by subunit-specific AMPAR interacting proteins as well as by various post-translational modifications that occur on their cytoplasmic carboxyl terminal (C-terminal) domains. The regulated trafficking of AMPARs is a major mechanism underlying activity-induced changes in synaptic transmission (reviewed in Derkach et al., 2007, Shepherd and Huganir, 2007, Santos et al., 2009 and Anggono and Huganir, 2012).

AMPAR structure and subunit composition

AMPARs are assembled by the association of two dimers in tetramers (Rosenmund et al., 1998; Sobolevsky et al., 2009) composed of one (homomeric) or two (heteromeric) of the four AMPARs subunits (reviewed in Traynelis et al., 2010). At mature hippocampal excitatory synapses, AMPARs consist predominantly of GluA1/GluA2 and, in some cases, of GluA2/GluA3 subunits (Wenthold et al., 1996; reviewed in Kauer and Malenka, 2006). AMPARs subunits GluA1-GluA4 combine in different stoichiometries (Hollmann and Heinemann, 1994) to form ion channels with distinct functional properties. It should be noted that the presence of the GluA2 subunit has a profound impact on the properties of AMPARs heteromeric complexes. As indicated above, in the adult brain GluA2 mRNA is subjected to RNA editing such that the GluA2 protein contains an arginine (R) residue within a reentrant membrane loop region at position 586 in place of genomically-encoded glutamine (Q) (Sommer et al., 1991). This Q/R editing controls various AMPARs properties including Ca^{2+} permeability, channel conductance,

kinetics and receptor affinity for glutamate, and subunit assembly into a functional receptor (Geiger et al., 1995; Swanson et al., 1997; Dingledine et al., 1999; Mansour et al., 2001; Greger et al., 2003; Oh and Derkach, 2005). AMPARs subunits have extracellular and transmembrane regions very similar but vary in their intracellular cytoplasmic tails. The GluA1, GluA4, and an alternative splice form of GluA2 (GluA2L) have longer cytoplasmic tails with high homology (Figure 19). In contrast, the predominant splice form of GluA2, GluA3, and an alternative splice form of GluA4 (Glu4c) have shorter, homologous cytoplasmic tails (Figure 19). The subunit composition of AMPARs also governs the rules of AMPARs trafficking. Receptors with subunits containing short cytoplasmic termini (GluA2/3) continuously cycle in and out of the synapse, while those with long cytoplasmic tails (GluA1/2 and GluA2/4) are driven into synapses by synaptic activity (Hayashi et al., 2000; Zhu et al., 2000; Passafaro et al., 2001; Shi et al., 2001). Transmembrane AMPA receptor regulatory proteins (TARPs), in particular stargazin, also coassemble stoichiometrically with native AMPARs (reviewed in Nicoll et al., 2006, Ziff, 2007 and Jackson and Nicoll, 2011). Stargazin and its closely related γ-3, γ-4 and γ-8 paralogs act as auxiliary subunits, interacting directly with all the AMPARs subunits to promote their transport to the cell surface (Chen et al., 2000; Tomita et al., 2003) and to modulate channel function (Priel et al., 2005; Tomita et al., 2005a).

Long tail



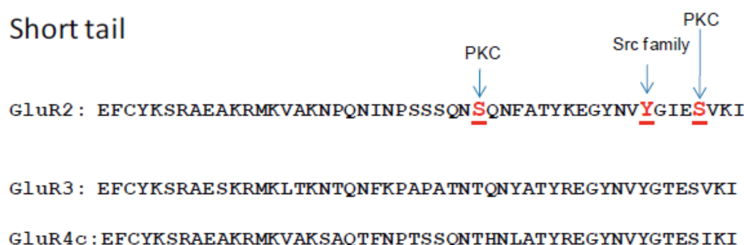


Figure 19. Schematic representation of the AMPA receptor subunits intracellular cytoplasmic tails. Sequence alignment of the intracellular C-terminal regions of the long-tailed (GluA1, GluA2L and GluA4) and short-tailed (GluA2, GluA3 and GluA4c) AMPA receptor subunits. The phosphorylation sites are underlined and indicated with a larger font size. [Reproduced from (Santos et al., 2009)].

As referred above AMPARs form tetramers, with each subunit presenting a characteristic modular organization consisting of four discrete domains (reviewed in Wo and Oswald, 1995, Mayer, 2006 and Traynelis et al., 2010): i) a large extracellular amino-terminal

domain (ATD) that participates in subunit-specific receptor assembly and modulation; ii) a ligand-binding domain (LBD); iii) a transmembrane domain (TMD) composed of three membrane-spanning segments (M1, M3 and M4) plus a short reentrant loop (M2) that forms the ion channel; iv) three intracellular domains, Loop1, Loop2 and the carboxyl-terminal domain (C-tail), highly variable in length. C-tails of the AMPA receptor subunits have long been shown to play crucial roles in receptor trafficking and function, as well as in determining subcellular localization, and in receptor coupling to intracellular signaling complexes (reviewed in Malinow and Malenka, 2002, Bredt and Nicoll, 2003 and Shepherd and Huganir, 2007).

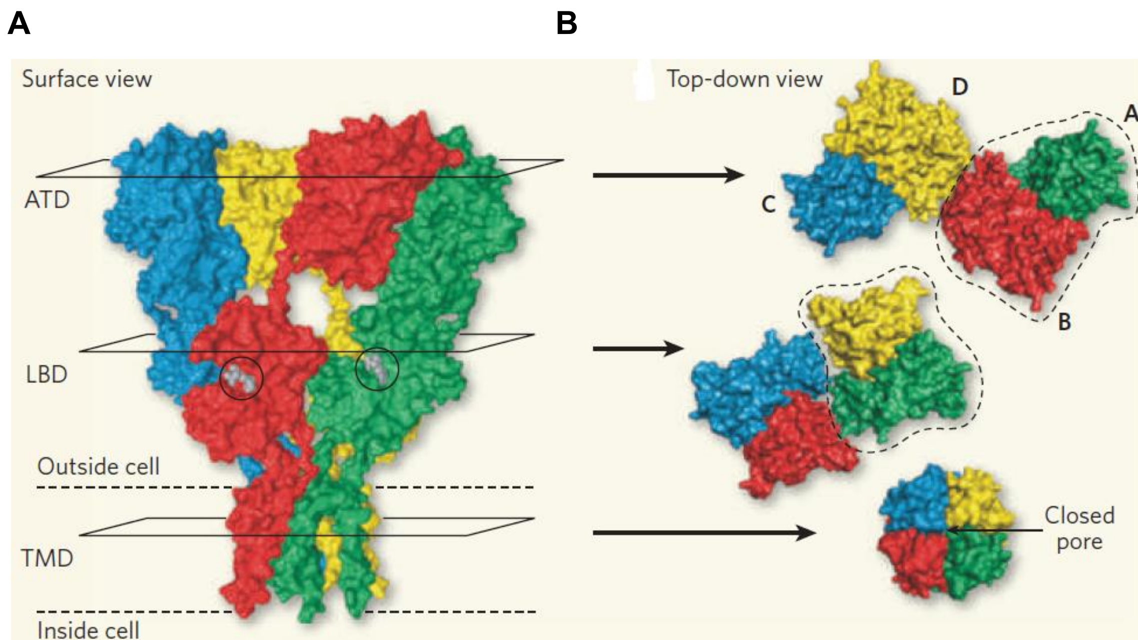


Figure 20. The tetrameric structure of the AMPA GluA2 receptor. (**A**) X-ray crystal structure of the AMPA GluA2 homotetrameric receptor (Sobolevsky et al., 2009). Each subunit is in a different color. The tetramer shows a typical layer organization with at the “top” the amino-terminal domains (ATDs), at the “bottom” the transmembrane domain (TMD) where the ion channel sits, and sandwiched between the two the agonist-binding domains (LBDs) binding glutamate. (**B**) Top-down view of the ATD, LBD and TMD, illustrating the domain swapping and symmetry mismatch (between LBD and TMD). The dashed line for the ATD and LBD indicates the dimer containing the A subunit, which in the ATD associates with the B subunit, but in the LBD associates with the D subunit. The TMD shows four-fold symmetry. Because the LBD is bound by an antagonist, the permeation pore, located in the centre of the TMD, is closed. [Reproduced from (Wollmuth and Traynelis, 2009)].

Crystallografic studies have provided the first detailed structure of a membrane-spanning glutamate receptor (Sobolevsky et al., 2009) (Figure 20). This structure of an antagonist-bound homomeric GluA2 receptor demonstrates that the receptor has an overall 2-fold symmetry perpendicular to the membrane plane. The extracellular ATDs and LBDs are organized as two dimers (i.e., each domain partners with the neighboring domain, forming two pairs of local dimers – 2-fold symmetry), whereas the pore region exhibits the typical 4-fold symmetry. Accompanying this symmetry mismatch another key feature revealed by the GluA2 structure was the domain swapping. Thus, if at the ATD level subunit A pairs with subunit B

(A/B dimer) and subunit C with D (C/D dimer), at the LBD level the two pairs engage different subunits (A/D and B/C dimers). This pairwise arrangement is abolished in the TMD, in which four independent but equivalent subunits have 4-fold symmetry. Crossover of subunit pairing likely helps to stabilize the large and loosely packed extracellular region (reviewed in Wollmuth and Traynelis, 2009).

Regulation of AMPARs function by phosphorylation

Two distinct but interrelated mechanisms regulate AMPAR function: modulation of the receptor ion channel properties (e.g., Derkach et al., 1999) and regulation of the synaptic targeting of the receptor (e.g., Hayashi et al., 2000). Both of these processes are regulated by receptor phosphorylation, in particular in their intracellular C-terminal region (reviewed in Henley et al., 2011 and Lu and Roche, 2012) (Figure 19). Interestingly, AMPARs phosphorylation is also considered an important event for signaling pathways associated with synaptic plasticity and learning (Lee et al., 2003; Whitlock et al., 2006).

Phosphorylation of serine 831 (Ser^{831}) in the intracellular C-terminus of GluA1 by calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) (Barria et al., 1997; Mammen et al., 1997) significantly increases single-channel conductance of homomeric GluA1 AMPARs (Derkach et al., 1999), by enhancing the coupling efficiency between glutamate binding and channel opening (Erreger et al., 2004). Roche and co-workers found that this residue is also phosphorylated by protein kinase C (PKC) (Roche et al., 1996). In native AMPARs the presence of the GluA2 subunit disrupts the coupling of Ser^{831} phosphorylation to channel conductance in GluA1/GluA2 heteromers, and this keeps receptors in a low-conductance state regardless of Ser^{831} phosphorylation (Oh and Derkach, 2005). However, this effect can be restored by coexpression of GluA1/A2 heteromers with TARPs (Kristensen et al., 2011). Additionally, the phosphorylation in this residue does not seem to be required for receptor synaptic delivery, since mutation of Ser^{831} to alanine, which prevents its phosphorylation by CaMKII, does not prevent delivery of receptors to synapses by active CaMKII (Hayashi et al., 2000).

It was also found that the intracellular C-terminus of GluA1 is phosphorylated by protein kinase A (PKA) (Roche et al., 1996) and cGMP-dependent kinase II (cGKII) (Serulle et al., 2007) in serine 845 (Ser^{845}). The AMPARs (homomeric GluA1) mean open probability is increased by PKA through phosphorylation of Ser^{845} (Banke et al., 2000). Additionally, the phosphorylation in this residue was also implicated in trafficking of AMPARs. Esteban and co-workers found that mutation of Ser^{845} to alanine, in opposition to Ser^{831} (Hayashi et al., 2000), does prevent delivery of GluA1 to synapses by active CaMKII (Esteban et al., 2003), suggesting that PKA phosphorylation of GluA1 at Ser^{845} is required for synaptic incorporation of AMPARs by CaMKII. On the other hand, PKA activity is necessary but not sufficient for the CaMKII-driven incorporation of GluA1 into synapses (Esteban et al., 2003). The authors suggested that PKA-dependent phosphorylation of GluA1 at Ser^{845} together with CaMKII activity are both required for

the synaptic incorporation of GluA1-containing AMPARs. This idea had already been proposed by Hayashi and co-workers, who suggested that some protein(s) other than GluA1 must be substrate(s) of CaMKII and participate in the regulated synaptic delivery of AMPARs (Hayashi et al., 2000). Nevertheless, Ser^{845} phosphorylation may also induce AMPARs delivery to the extrasynaptic membrane and then to the synapse via synaptic NMDARs activation (i.e., Ser^{845} phosphorylation primes AMPARs for synaptic insertion by trafficking them to extrasynaptic sites) (Sun et al., 2005; Oh et al., 2006). In addition, it was found that upon NMDARs activation AMPARs are sorted in early endosomal compartments (accompanied by dephosphorylation of GluA1 at Ser^{845}), and then selectively targeted for plasma membrane reinsertion, mediated by PKA activity through rephosphorylation of Ser^{845} (Ehlers, 2000), also suggesting an activity-dependent process for AMPARs synaptic trafficking. Consistently with the role of Ser^{845} phosphorylation in the trafficking of GluA1-containing AMPARs to plasma membrane, Serule and co-workers found that cGKII binds the GluA1 intracellular C-terminus in a cGMP-dependent manner, and that in this complex, cGKII can phosphorylate Ser^{845} of GluA1 and increase GluA1 levels in extrasynaptic sites (Serulle et al., 2007).

Two other serine residues located in the membrane proximal region (MPR) of the intracellular C-terminus of the GluA1 subunit were implicated in the synaptic incorporation of GluA1-containing AMPARs. Boehm and co-workers found that serine 818 (Ser^{818}) is phosphorylated by PKC, promoting GluA1 synaptic incorporation (Boehm et al., 2006). Indeed, mimicking phosphorylation at Ser^{818} together with Ser^{831} and Ser^{845} allows GluA1 accessing to the synapse (Boehm et al., 2006). Later, it was confirmed that the phosphorylation of Ser^{818} by PKC is implicated in GluA1-containing AMPARs trafficking (Lin et al., 2009). These authors found that phosphorylation in this residue together with the phosphorylation in serine 816 (Ser^{816}), by PKC, enhanced the binding of protein 4.1N to GluA1 facilitating GluA1 insertion in surface extrasynaptic pools (Lin et al., 2009). This protein had already been implicated in AMPARs trafficking: 4.1N is an actin-binding protein which binds GluA1 (in the MPR region of the intracellular C-terminal), stabilizing its surface expression (Shen et al., 2000). The binding of 4.1N to GluA1 is regulated by palmitoylation (reversible fatty acid acylation) of GluA1. Hayashi and co-workers found that a C-terminal palmitoylation site (cysteine 811 – Cys^{811}) overlaps with the region of the AMPA receptor subunits that binds the cytoskeletal protein 4.1N (Hayashi et al., 2005). Palmitoylation on the C-terminal domain of GluA1 inhibits its interaction with the 4.1N protein (Hayashi et al., 2005). Palmitoylation acts by restricting the access of PKC to Ser^{816} and Ser^{818}, thus depalmitoylation of GluA1 Cys^{811} leads to PKC phosphorylation in these residues, which in turn enhances the interaction between 4.1N and GluA1 and results in increased GluA1 surface insertion (Lin et al., 2009). Altogether, these results suggest that the interplay between protein palmitoylation and phosphorylation may play important roles in the postsynaptic trafficking of AMPARs and in the regulation of excitatory synaptic transmission.

In addition to serine residues, it was found that GluA1 intracellular C-terminal region is phosphorylated by PKC (Lee et al., 2007) and p70S6 kinase (Delgado et al., 2007) at threonine 840 (Thr^{840}). Thr^{840} is one of the major phosphorylation sites on GluA1 and shows rapid turnover under basal conditions in the hippocampus, sustained by a balance between persistently active protein kinase(s) and protein phosphatase(s) (Lee et al., 2007); this residue is also rapidly dephosphorylated after activation of NMDARs by protein phosphatases 1 and/or 2A (Delgado et al., 2007).

In addition to the multiple phosphorylation sites in the GluA1 intracellular C-terminal region, recently the GluA1 Loop1 domain was shown to be phosphorylated on serine 567 (Ser^{567}). Biochemical studies demonstrated that CaMKII phosphorylates Ser^{567} (Lu et al., 2010). The GluA1 Loop1 itself facilitates AMPA receptor targeting to synapses, and phosphorylation at Ser^{567} negatively modulates Loop1-dependent synaptic delivery of AMPA receptors (Lu et al., 2010). Therefore, a dual mechanism mediated by GluA1 Loop1 and Ser^{567} phosphorylation controls synaptic targeting of GluA1-containing receptors (Lu et al., 2010).

GluA4 is expressed in the hippocampus during the early postnatal period, and spontaneous neuronal activity at this stage is sufficient to trigger GluA4 synaptic incorporation (Zhu et al., 2000). Phosphorylation of GluA4 at serine 842 (Ser^{842}) by PKA (this residue is also phosphorylated in *vitro* by PKC and CaMKII) (Carvalho et al., 1999), is both necessary and sufficient for the delivery of GluA4-homomeric receptors to the synapse (Esteban et al., 2003). Threonine 830 (Thr^{830}) was also identified as a potential PKC phosphorylation site (Carvalho et al., 1999). In addition, PKC isoform γ (PKCγ) interacts directly with GluA4 in the MPR and phosphorylates Ser^{842} *in vitro* (Correia et al., 2003) and in cultured retina cells (Gomes et al., 2004). Disruption of this interaction prevents efficient receptor phosphorylation by PKCγ and the PKCγ-driven increase in cell surface expression of GluA4-containing AMPARs (Gomes et al., 2007).

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 Henley, 2003 and Lee and Zheng, 2010). Of particular interest, PKC phosphorylates the GluA2 subunit in one of these motifs (IESVKI), in serine 880 (Ser^{880}), through which GluA2 binds to different proteins - e.g., glutamate receptor interacting protein (GRIP)/ABP and protein interacting with C-kinase-1 (PICK1) - therefore modulating those interactions (Matsuda et al., 1999, 2000; Chung et al., 2000; Perez et al., 2001; Seidenman et al., 2003). PKC also phosphorylates GluA2 in serine 863 (Ser^{863}) (McDonald et al., 2001), however the function of the phosphorylation of this residue is still not known. Phosphorylation of GluA2 (Ser^{880}) by PKC, which prevents the association of GluA2 with GRIP (Matsuda et al., 1999, 2000; Chung et al., 2000) but promotes binding to PICK1 (Matsuda et al., 1999; Chung et al., 2000; Perez et al., 2001), thus recruits PICK1 to excitatory synapses and

facilitates rapid internalization of surface receptors. Phosphorylation of Ser^{880} promotes internalization of AMPARs and decreases surface GluA2-containing receptors in both Purkinje neurons (Matsuda et al., 2000) and hippocampal neurons (Chung et al., 2000; Perez et al., 2001).

GluA2 is also phosphorylated at tyrosine 876 (Tyr^{876}) in its intracellular C-terminal by the Src family protein tyrosine kinases, *in vitro* and *in vivo* (Hayashi and Huganir, 2004). Interestingly, phosphorylation of this tyrosine residue has similar effects to the phosphorylation of Ser^{880}. Phosphorylation of GluA2 on Tyr^{876} decreases the binding to GRIP/ABP, but is without effect on the binding to PICK1, thus facilitating the AMPA- or NMDA-induced receptor internalization (Hayashi and Huganir, 2004). Finally, using bioinformatic and biochemical approaches, two sites of phosphorylation in threonine residues were identified in AMPA receptor GluR2L (Thr^{912}) and GluR4 (Thr^{855}) subunits by Jun N-terminal kinase 1 (JNK1) (Thomas et al., 2008). It was found that the JNK site in GluR2L controls reinsertion of internalized GluR2L back to the cell surface following NMDA treatment, without affecting basal GluR2L trafficking (Thomas et al., 2008).

Regulation of AMPARs trafficking by interacting proteins and phosphorylation

Extrasynaptic insertion of AMPARs

The number of AMPARs at the synapse is dependent on relative rates of exocytosis and endocytosis at the postsynaptic membrane. Enhanced receptor exocytosis and recycling occurs during synaptic potentiation, while increased rate of endocytosis results in synaptic depression (reviewed in Shepherd and Huganir, 2007 and Kessels and Malinow, 2009). AMPARs are inserted into the plasma membrane in the soma or dendrites at extrasynaptic sites and travel to dendritic spines via lateral diffusion (Adesnik et al., 2005; Yudowski et al., 2007; Lin et al., 2009). The synaptic targeting of AMPARs is largely regulated by both AMPARs interaction partners and phosphorylation (reviewed in Henley et al., 2011 and Anggono and Huganir, 2012).

In the absence of neuronal activity, AMPARs undergo constitutive recycling between synapses and the cytosol, where they are sorted for either degradation or reinsertion at synapses (Ehlers, 2000; Passafaro et al., 2001). Upon synaptic potentiation they are more actively recycled through an endossomal pathway to enhance exocytosis (Park et al., 2004). Some observations support the idea that AMPARs do not directly exchange between the postsynaptic density (PSD) and cytosolic compartments. Instead, exocytosis and endocytosis appear to occur at extrasynaptic membrane sites from which AMPARs laterally diffuse into and out of the synaptic PSD.

Passafaro and co-workers found that GluA1-containing AMPARs in the hippocampus are inserted initially at extrasynaptic sites, followed by their slow incorporation into synapses, upon NMDARs activation (Passafaro et al., 2001). Interestingly, stimulation of D1 dopamine receptors, in the prefrontal cortex, results in the PKA-dependent clustering of GluA1-containing AMPARs near, but not whithin the PSD (Sun et al., 2005). Subsequent NMDARs activation promotes synaptic insertion of AMPARs. These findings suggest that in two different brain regions (hippocampus and prefrontal cortex), GluA1-containing AMPARs are first delivered to extrasynaptic sites before incorporation into synapses, and that this last step is mediated by NMDARs activation. This assumptiom was confirmed by Oh and co-workers, who also found that this trafficking pattern is correlated with the PKA-dependent phosphorylation of GluA1. They observed that receptors phosphorylated at Ser^{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 the following model: Ser^{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). The phosphorylation in this residue has been extensively implicated in AMPARs trafficking, as mentioned above (section: "*Regulation of AMPARs function by phosphorylation*"). For instance, NMDARs activation triggers AMPARs endocytosis (by direct dephosphorylation of GluA1 at Ser^{845}) and is followed by rapid, PKA-dependent AMPARs reinsertion via recycling endosomes, through re-phosphorylation in the Ser^{845} (Ehlers, 2000). cGKII also phosphorylates Ser^{845} of GluA1 increasing its clustering in the plasma membrane (Serulle et al., 2007). Altogether, this evidence points to the fact that phosphorylation in the Ser^{845} is a major event for GluA1-containing AMPARs synaptic insertion. Hence, we can conclude that two phenomena should occur for synaptic clustering: i) GluA1 phosphorylation (Ser^{845}) and ii) NMDARs activation.

Esteban and co-workers found that although activation of PKA alone by increasing intracellular cAMP did produce phosphorylation of GluA1, it was not sufficient for the synaptic incorporation of GluA1 (Esteban et al., 2003). Nevertheless, they observed that a GluA1 phosphodead mutant (with Ser^{845} replaced by alanine) when coexpressed with constitutively active CaMKII was not driven into synapses (Esteban et al., 2003), thus suggesting that PKA phosphorylation of GluA1 at Ser^{845} is required for synaptic incorporation of GluA1 by CaMKII. However, some protein(s) other than GluA1 must be substrate(s) of CaMKII and participate in the regulated synaptic delivery of AMPARs, because the substitution of the serine residue in GluA1, which is substrate for CaMKII, did not block the CaMKII-dependent synaptic delivery of GluA1 (Hayashi et al., 2000). Additionally, these authors found that mutating a predicted PDZ domain interaction site in GluA1 C-terminus blocked the CaMKII-dependent synaptic delivery of GluA1 (Hayashi et al., 2000), suggesting that CaMKII-dependent phosphorylation of an unidentified substrate containing a PDZ domain-binding site is required for GluA1 synaptic clustering.

Hence, incorporation of GluA1-containing AMPARs into synapses requires: i) phosphorylation of GluA1 (which mediates the extrasynaptic clustering), and ii) CaMKII activity (stabilizing synaptically delivered receptors) upon NMDARs activation. Conspicuous evidence suggests that an important substrate of CaMKII in this process is the AMPARs-associated protein stargazin (Chen et al., 2000; Tomita et al., 2005b; Opazo et al., 2010); however the interaction between AMPARs and stargazin does not depend on the C-terminal domain of GluA1 (reviewed in Jackson and Nicoll, 2011), and consequently this is not the substrate suggested by Hayashi and co-workers. Despite this, these findings suggest, unequivocally, that two independent phenomena are required for synaptic clustering to occur, as indicated above, GluA1 phosphorylation and NMDARs activation.

The mechanism by which stargazin stabilizes AMPARs at the synapse will be discussed in the next section ("*Synaptic clustering of AMPARs"*). However, even having these considerations in mind we should also refer that other authors have suggested that CaMKII triggers cellular machinery, mediated by Ras signaling (Zhu et al., 2002), that results in the synaptic insertion of AMPARs (reviewed in Esteban, 2003), and emphasize that synaptic targeting/insertion and synaptic stabilization may be mediated by several mechanisms (related with AMPARs phosphorylation and/or AMPARs interacting proteins) (extensively reviewed in Derkach et al., 2007, Shepherd and Huganir, 2007, Kessels and Malinow, 2009, Santos et al., 2009, Anggono and Huganir, 2012 and Opazo et al., 2012).

We should also refer briefly other aspects that have been implicated in GluA1-containing AMPARs trafficking. For instance, it was found that PKC-dependent phosphorylation of Ser^{816} and Ser^{818}, through the binding of 4.1N protein, enhanced GluA1 insertion to surface extrasynaptic pools (Lin et al., 2009). The PKC-dependent phosphorylation of Ser^{818} had been already related with synaptic incorporation of AMPARs (Boehm et al., 2006).

Altogether, these observations indicate that GluA1-containing AMPARs are probably trafficked to synapses in two steps: the first step, which occurs in the absence of synaptic stimulation and is associated with phosphorylation of GluA1, mobilizes receptors to extrasynaptic sites, whereas the second step involves NMDARs Ca^{2+} influx to enhance stabilization of receptors in the synapse, thus inducing synaptic potentiation (Figure 21).

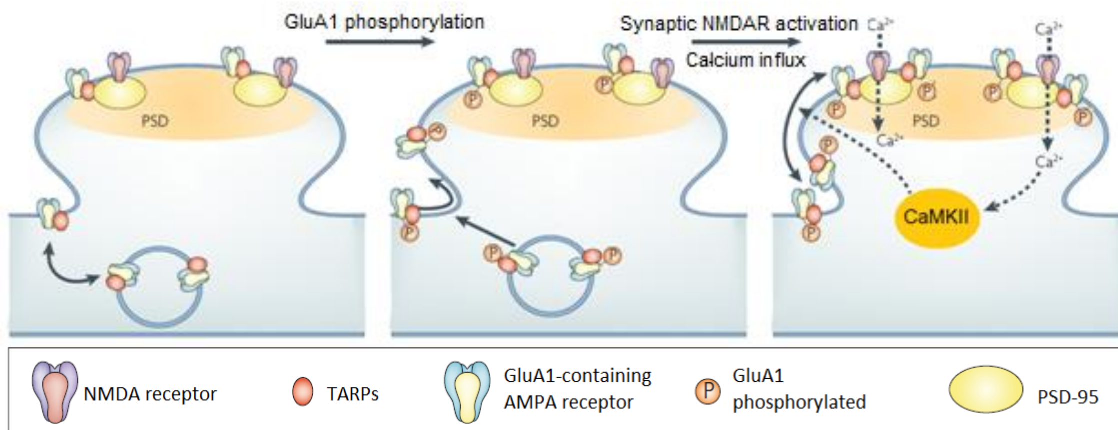


Figure 21. Multistep trafficking of AMPARs to synapses. *Left*, under basal conditions, AMPA receptors constitutively cycle to and from the synaptic membrane, dictated largely by the GluA2 subunit. *Middle*, Phosphorylation of Ser$^{845/816/818}$ in GluA1 is associated with the insertion of GluA1-containing AMPARs into extrasynaptic sites. *Right*, potentiation requires trafficking of these extrasynaptic AMPARs to synapses, triggered by Ca^{2+} influx through NMDA receptors and presumably Ca^{2+}-dependent signaling, including CaMKII. PSD-95, postsynaptic density protein 95. [Adapted from (Derkach et al., 2007)].

Synaptic clustering of AMPARs

At excitatory synapses, two of the major proteins that are dynamically recruited postsynaptically are CaMKII and AMPARs. Numerous studies have shown that CaMKII is involved in the activity and NMDARs-dependent synaptic recruitment of AMPARs both during synaptic development and synaptic plasticity (Pettit et al., 1994; Rongo and Kaplan, 1999; Hayashi et al., 2000; Lisman et al., 2002; Poncer et al., 2002; Merrill et al., 2005; Asrican et al., 2007; Sanhueza et al., 2007; Zhang et al., 2008; Lee et al., 2009). Two principal recruitment mechanisms can be anticipated: CaMKII might promote the exocytosis of AMPARs-containing vesicles (Maletic-Savatic et al., 1998) and/or the trapping at the PSD of laterally diffusing AMPARs (reviewed in Opazo et al., 2012).

Some observations have suggested that CaMKII can recruit AMPARs by diffusional trapping. First, NMDARs activation causes the rapid translocation of CaMKII from dendritic compartments to activated synapses (Strack et al., 1997; Shen and Meyer, 1999; Hudmon et al., 2005). Second, following NMDARs activation, CaMKII can remain at postsynaptic sites for prolonged periods of time, through binding to several PSD proteins, including the NMDARs (Lisman et al., 2002; Otmakhov et al., 2004; Bayer et al., 2006). Third, CaMKII bound to the NMDARs remains active independent of Ca^{2+}/CaM (Bayer et al., 2001), which should allow it to phosphorylate incoming membrane-bound proteins. Fourth, AMPARs are highly mobile at the neuronal surface, rapidly switching between extrasynaptic and synaptic sites (Borgdorff and Choquet, 2002; Tardin et al., 2003; Bats et al., 2007; Heine et al., 2008). Fifth, NMDARs stimulation, high-frequency stimulation, or increases in intracellular Ca^{2+} all promote the rapid immobilization of AMPARs (Borgdorff and Choquet, 2002; Heine et al., 2008; Makino and Malinow, 2009; Petrini et al., 2009).

However the most striking evidence came out very recently. Opazo and co-workers found in hippocampal neurons that, upon NMDARs activation, CaMKII activation stops the diffusion of surface AMPARs at synaptic sites (Opazo et al., 2010). Futhermore they showed that the function of CaMKII is mediated by phosphorylation of stargazin and binding of its C-terminus to PDZ domain of the scaffold protein PSD-95, thus indirectly stabilizing AMPARs at synapses (Opazo et al., 2010). Taken together, these findings suggest a scenario in which AMPARs are intrinsically mobile at the neuronal surface and can be ‘‘trapped’’ at activated synapses in a CaMKII-dependent manner (Figure 22).

The C-terminal domain of stargazin has a series of nine conserved serines, found within a highly basic region, which are substrates for phosphorylation by CaMKII and/or PKC (Tomita et al., 2005b). In aggreement with Opazo and co-workers (Opazo et al., 2010), it was found that expression of a phosphomimetic stargazin construct enhances AMPARs-synaptic currents, an effect which is lost when expressing a construct lacking the C-terminal PDZ binding site (Tomita et al., 2005b), thus indicating that the stargazin PDZ binding domain (which is bound by PSD-95) is necessary for synaptic trafficking of AMPARs by phosphorylated stargazin.

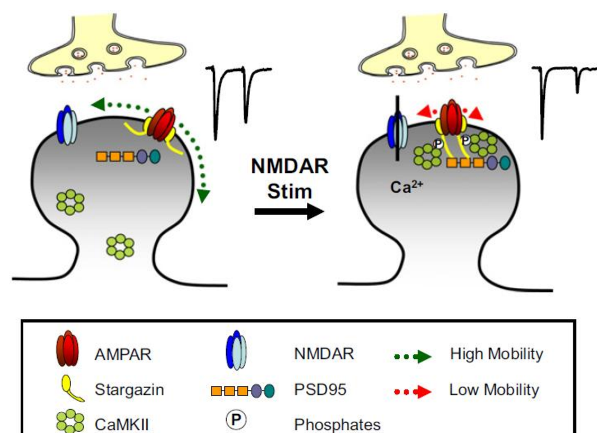


Figure 22. **Model of NMDARs-dependent trapping of laterally diffusing AMPARs**. At basal state, AMPARs are highly mobile at the neuronal surface, rapidly exchanging between extrasynaptic and synaptic sites, while CaMKII is not enriched in the PSD. Upon NMDARs activation, Ca^{2+}/CaM activates CaMKII and induces its postsynaptic translocation. In the PSD, CaMKII phosphorylates the AMPARs auxiliary protein stargazin, facilitating its binding to PSD-95 and synaptic retention, thereby indirectly stabilizing AMPARs at synapses. [Reproduced from (Opazo et al., 2010)].

AMPARs and synaptic plasticity

One of the most remarkable features of the brain is its ability to store vast amounts of information. Changes in the strength of synaptic connections as a mechanism underlying learning and memory had been proposed by Cajal at the beginning of the last century and then formulated into a concrete synaptic model by Hebb in 1949 (Hebb, 1949). However, it was not until the discovery of long-term potentiation (LTP) by Bliss and Lomo (Bliss and Lomo, 1973), in

which brief high frequency synaptic stimulation in the hippocampus results in a long lasting increase in synaptic strength, that experimental evidence for such a proposal was obtained.

Changes in synaptic strength are thought to underlie memory storage in the brain (reviewed in Martin et al., 2000). LTP and long-term depression (LTD) are the two most-studied and prevailing cellular models of synaptic plasticity (reviewed in Malenka and Bear, 2004, Lüscher & Malenka, 2012 and Nicoll & Roche, 2013). LTP and LTD can be elicited by activating NMDARs, typically by the coincident activity of pre- and postsynaptic neurons. The expression of synaptic plasticity is mediated by a redistribution of AMPARs: more receptors are added to potentiate the synapse or receptors are removed to weaken synapses (Figure 23). With time, structural changes become apparent, which in general require the synthesis of new proteins.

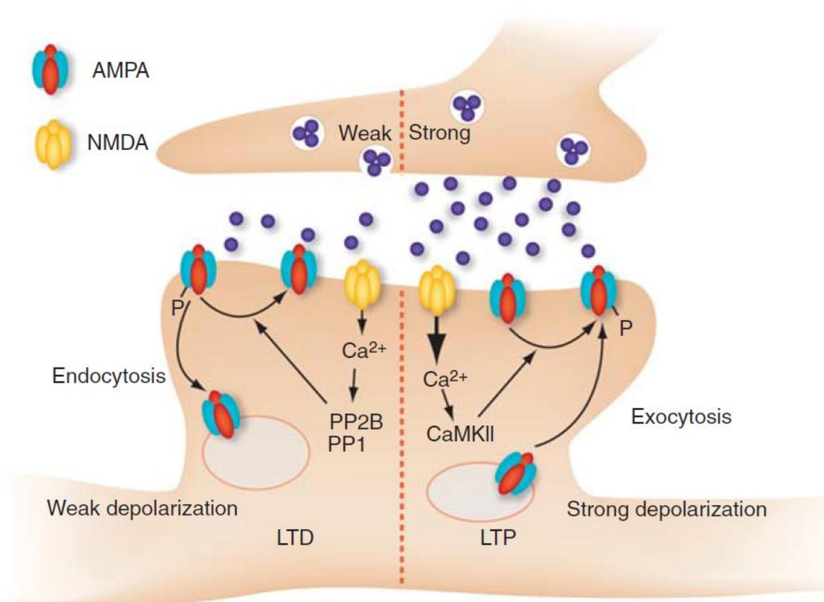


Figure 23. **Postsynaptic expression mechanisms of LTP and LTD**. Weak activity of the presynaptic neuron leads to modest depolarization and calcium influx through NMDA receptors. This preferentially activates phosphatases that dephosphorylate AMPA receptors, thus promoting receptor endocytosis. Strong activity paired with strong depolarization triggers LTP in part via CaMKII, receptor phosphorylation, and exocytosis. [Reproduced from (Lüscher and Malenka, 2012)].

Long-term potentiation

Several lines of evidence point to a central role of GluA1 in hippocampal LTP. For instance, mature GluA1-knockout mice lack LTP in the CA1 region of the hippocampus (Zamanillo et al., 1999), and the defect is rescued by genetically expressing GluA1 (Mack et al., 2001). Studies using hippocampal organotypic hippocampal slices transiently expressing GFP-tagged AMPARs subunits showed a rapid translocation of GluA1-GFP to dendritic spines following induction of LTP (Shi et al., 1999; Hayashi et al., 2000). The translocation of GluA1 to the membrane requires high-frequency stimulation of the synapse and is dependent on NMDARs activation (Shi et al., 1999). Moreover, it was found that it requires the association between GluA1 and an unidentified PDZ domain protein (Hayashi et al., 2000).

The experimental strategy based in GFP-tagged AMPARs, which allows the formation of homomeric channels, is very useful for the study of subunit specificity in trafficking; however it does not address the regulation of endogenous heteromeric channels. In agreement with the previous findings, the endogenous AMPARs can also be driven into the synapse during LTP (Shi et al., 2001) and experience (Takahashi et al., 2003). Insertion requires the C-terminal region, because expression of the GluA1 cytoplasmic tail prevents LTP (Shi et al., 2001) and the experience-dependent delivery of AMPARs (Takahashi et al., 2003).

Moreover, it was proposed that GluA1-GluA2 receptors are excluded from synapses unless an LTP stimulus is provided, whereas GluA2-GluA3 receptors traffic to the synapse constitutively (Shi et al., 2001). This difference in trafficking behavior is mediated by the C-tails of the individual subunit proteins. In agreement with this model, LTP is normal in GluA2-GluA3 double knockouts (Meng et al., 2003).

Hence, a broad consensus has emerged arguing that LTP is mediated by synaptic insertion of GluA1-containing AMPARs via its C-tail (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Malenka, 2003; Collingridge et al., 2004; Malenka and Bear, 2004; Shepherd and Huganir, 2007; Kessels and Malinow, 2009; Anggono and Huganir, 2012). However, some evidence indicates that GluA1-independent LTP also exists. Mature GluA1-knockout mice show some LTP in the dentate gyrus (Zamanillo et al., 1999) and also show LTP in the CA1 region in young mice (Mack et al., 2001).

In fact, very recently it was found that the GluA1 C-tail is not required for LTP in the CA1 region of the hippocampus (Granger et al., 2013), in striking contrast with the prevailing model. To define the minimal requirements for LTP these authors have used a single molecular replacement strategy, in which endogenous AMPARs are deleted by expressing Cre in neurons from triple floxed mice (Gria1$^{fl/fl}$ Gria2$^{fl/fl}$ Gria3$^{fl/fl}$), and then mutated forms of AMPAR subunits were re-introduced onto this null background. It was found that no region in the GluA1 C-tail was essential either for basal synaptic incorporation or for LTP (Granger et al., 2013). In fact, neurons expressing homomeric GluA2 receptors exhibited normal LTP, and surprisingly, hippocampal synapses in which AMPARs had been replaced with kainate-type glutamate receptors also expressed normal LTP (Granger et al., 2013). The only conditions under which LTP was impaired were those with dramatically decreased AMPARs surface expression, indicating a requirement for a reserve pool of receptors (Granger et al., 2013), suggesting that synapses are remarkable flexible in order to be potentiated.

However, these findings should be interpreted with some caution, because genetically deleting all AMPARs may fundamentally change AMPARs trafficking compared with control conditions (Sheng et al., 2013). For instance, GluA1 subunits lacking their C-tail could reach synapses only when all AMPARs were deleted and not in the control neurons, in which other

AMPARs are present (Sheng et al., 2013). To address the mechanisms underlying LTP at fully equipped synapses, more subtle experimental manipulations may be more informative; these could include introducing mutations (“knock-in” mutations) into the genes encoding GluA1 and GluA2 to alter their C-tail structure without affecting their expressing levels (Sheng et al., 2013).

In fact, Lee and co-workers found that mice with such mutations (Ser^{831} and Ser^{845} mutated to alanine) show reduced LTP in the CA1 region of hippocampus, as well as defected retention of a spatial memory task using the Morris water maze test (Lee et al., 2003), suggesting that phosphorylation of the receptor GluA1 subunit is required for LTP and spatial memory formation. Accordingly, CaMKII, PKA and PKC activity, through the phosphorylation of their target residues in the GluA1 C-terminal domain (in particular, Ser^{831} and Ser^{845}), mentioned in detail in the two previous sections, and which affect the AMPARs trafficking, as well as GluA1 interacting proteins have been extensively implicated in the induction and expression of LTP. Moreover, other signaling pathways have been also related with these processes, such as the Ras and PI3 kinase pathways (reviewed in Shepherd and Huganir, 2007, Santos et al., 2009, Anggono and Huganir, 2012 and Lüscher and Malenka, 2012).

Long-term depression

Low levels of synaptic stimulation can activate NMDARs to produce NMDARs-dependent LTD of glutamatergic synaptic transmission, or activate mGluRs to produce mGluRs-dependent LTD. The two forms of LTD are thought to result from internalization of surface AMPARs (Lissin et al., 1998; Beattie et al., 2000; Snyder et al., 2001; Xiao et al., 2001) in both hippocampal pyramidal neurons (NMDARs-dependent LTD) and cerebellar Purkinje cells (mGluRs-dependent LTD) (reviewed in Shepherd and Huganir, 2007 and Santos et al., 2009). Here, we will devote our attention to NMDARs-dependent LTD.

Regulation of the phosphorylation of AMPAR subunits is important for LTD expression. During hippocampal LTD, the PKA phosphorylation site on GluA1 (Ser^{845}) is dephosphorylated, whereas LTD induction in previously potentiated synapses leads to dephosphorylation of the CaMKII phosphorylation site (Ser^{831}) (Lee et al., 2000). Mice that have these two sites mutated also exhibit major deficits in LTD and AMPARs internalization induced by NMDARs activation (Lee et al., 2003).

The interaction of GluA2 with several proteins has also been implicated in LTD. It was found that, similar to what has been suggested in cerebellar LTD, disruption of the GluA2-GRIP/ABP interaction by GluA2 phosphorylation (at Ser^{880}, mediated by PKC) results in the removal of synaptic receptors, by facilitating the interaction between GluA2 and PICK1 (Chung et al., 2000; Perez et al., 2001). This was further supported by two different studies. In hippocampal slice cultures it was found that by mimicking GluA2 Ser^{880} phosphorylation receptors are excluded from synapses, to depress transmission, and LTD is partially occluded

(Seidenman et al., 2003). Moreover, it was found that a mutant form of PICK1, unable to bind lipids, impairs expression of LTD in hippocampal neurons (Jin et al., 2006).

However, other authors have found that although hippocampal LTD is accompanied by phosphorylation of Ser^{880}, PKC does not appear to mediate phosphorylation in this residue and hippocampal LTD does not seem to require PKC activity (Kim et al., 2001). In particular, these findings suggest that other kinases in the hippocampus may phosphorylate this residue. To increase this controversy, Daw and co-workers found that Ser^{880} phosphorylation can reverse LTD, by disrupting the intracellular retention of GluA2 by GRIP/ABP, and allowing receptors to be delivered to the synapse (Daw et al., 2000). Finally, it was found that changes in the phosphorylation state of the Ser^{880} residue at the C-terminus of GluA2 do not affect NMDARs-dependent GluA2 internalization, but alter the recycling of GluA2 after NMDAR activation (Lin and Huganir, 2007).

In conclusion, some controversy exists over the precise roles of GRIP and PICK1 in AMPAR trafficking and plasticity, and the exact understanding of their molecular functions is still missing. Some of the confusion on their role in LTD may be due to cell-type-specific differences. In addition, each protein may play multiple roles in the delivery, stabilization, and removal of synaptic AMPARs (reviewed in Shepherd and Huganir, 2007).

Objectives of the Present Study

Ghrelin was purified from the rat stomach in 1999 (Kojima et al., 1999). Since its discovery intensive research has been performed on ghrelin, and it is now well established the significance of ghrelin as a growth hormone-releasing hormone, appetite regulator and energy conservator (reviewed in Kojima and Kangawa, 2010). Currently, ghrelin is the only known circulating orexigenic hormone secreted from a peripheral organ that acts on the hypothalamic arcuate nucleus, which is the regulatory region of appetite. However, recent evidence has suggested that ghrelin originated in the periphery may also control higher brain functions and may represent a molecular link between learning capabilities and energy metabolism (reviewed in Andrews, 2011).

Indeed, studies from different laboratories have shown that endogenous ghrelin has a physiological role in improving learning and memory performance. However, the precise molecular mechanisms that underlie the effects of ghrelin on hippocampal function remain to be elucidated. The overall objective of this work was to test the hypothesis that ghrelin may affect excitatory transmission in the hippocampus. Therefore we pursued the following objectives:

1) We investigated the subcellular localization of the ghrelin receptor in hippocampal cultured neurons relatively to glutamatergic excitatory synapse, as well as its presence in synaptic fractions purified from adult hippocampi.

2) We examined the role of ghrelin receptor activation in excitatory synaptic transmission by performing electrophysiological recordings from organotypic hippocampal slices to analyze the synaptic recruitment of GluA1 in CA1 neurons, and the consequent functional modifications, namely in terms of AMPARs-mediated synaptic currents.

3) We determined whether ghrelin receptor activation increases the expression of NMDARs-dependent long-term potentiation in organotypic hippocampal slice cultures, as well as the synaptic trafficking of GluA1-containing AMPARs upon induction of chemical LTP in hippocampal cultured neurons.

4) We analyzed whether synaptic potentiation of AMPARs-mediated synaptic transmission, upon ghrelin receptor activation in the hippocampus, was accompanied by post-translational modifications in AMPARs, as well as in the AMPARs-associated protein stargazin, which are among the main molecular targets for the induction of AMPARs synaptic trafficking.

5) We analyzed the effects of ghrelin on hippocampal AMPARs traffic at different stages of development. In this context, we explored the contribution of ligand-independent ghrelin

receptor activity in AMPARs-mediated synaptic transmission, as well as in AMPARs and stargazin phosphorylation.

Our findings provide the first evidence pointing to a role of ghrelin in regulating the synaptic traffic of AMPAR in the hippocampus. This effect of ghrelin is likely to underlie its cognitive enhancing properties.

Chapter 2

Materials and Methods

Materials

Rat ghrelin, the GHS-R1a antagonist [D-Lys^{3}]-GHRP-6, TTX, AP5 and picrotoxin were purchased from Tocris Bioscience (Bristol, UK). The GHS-R1a agonist MK-0677 was purchased from Axon Medchem (Groningen, The Netherlands) and the GHS-R1a inverse agonist [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P from Bachem (Bubendorf, Switzerland). All other reagents were purchased from Sigma-Aldrich (Sintra, Portugal) or from Merck (Darmstadt, Germany) unless specified otherwise.

Primary Antibodies	Dilution (application)	Source
Actin	1:5000 (WB)	Sigma-Aldrich (Sintra, Portugal)
Akt	1:1000 (WB)	Cell Signaling Technology (Danvers, USA)
αCaMKII	1:2000 (WB)	Sigma-Aldrich (Sintra, Portugal)
GFP	1:500 (ICC)	MBL International (Woburn, USA)
GHS-R1a	1:200 (WB)	Millipore (Madrid, Spain)
GHS-R1a	1:100 (ICC)	Alpha Diagnostic International (Santo Antonio, USA)
GluA1	1:1000 (WB)	Millipore (Madrid, Spain)
GluA1	1:300 (ICC)	kind gift from Dr. Andrew Irwing (University of Dundee, Scotland)
MAP2	1:5000 (ICC)	Abcam (Cambridge, UK)
PSD-95	1:2000 (WB) 1:750 (ICC)	Cell Signaling Technology (Danvers, USA)
PSD-95	1:200 (ICC)	Affinity BioReagents (Golden, USA)
Synaptophysin	1:10000 (WB)	Abcam (Cambridge, UK)
Tubulin	1:200000 (WB)	Sigma-Aldrich (Sintra, Portugal)

P-Ser(PKC Substrates)	1:1000 (WB)	Cell Signaling Technology (Danvers, USA)
P-Ser239/240(Stargazin)	1:500 (WB)	Millipore (Madrid, Spain)
P-Ser473(Akt)	1:1000 (WB)	Cell Signaling Technology (Danvers, USA)
P-Ser831(GluA1)	1:5000 (WB)	Tocris Bioscience (Bristol,UK)
P-Ser845(GluA1)	1:1000 (WB)	Millipore (Madrid, Spain)
P-Thr286/287(α/βCaMKII)	1:1000 (WB)	Upstate (Millipore, Madrid, Spain)
Vglut1	1:5000 (ICC)	Millipore (Madrid, Spain)
Secondary Antibodies	**Dilution (application)**	**Source**
Alkaline phosphatase-conjugated anti-mouse	1:20000 (WB)	GE Heathcare (Carnaxide, Portugal)
Alkaline phosphatase-conjugated anti-rabbit	1:20000 (WB)	GE Heathcare (Carnaxide, Portugal)
Alexa 488-conjugated anti-mouse	1:500 (ICC)	Molecular Probes (Leiden, The Netherlands)
Alexa 488-conjugated anti-rabbit	1:500 (ICC)	Molecular Probes (Leiden, The Netherlands)
Alexa 488-conjugated anti-sheep	1:500 (ICC)	Molecular Probes (Leiden, The Netherlands)
Alexa 594-conjugated anti-rabbit	1:500 (ICC)	Molecular Probes (Leiden, The Netherlands)
Alexa 647-conjugated anti-guinea pig	1:500 (ICC)	Molecular Probes (Leiden, The Netherlands)
Texas red-conjugated anti-mouse	1:200 (ICC)	Molecular Probes (Leiden, The Netherlands)
AMCA-conjugated anti-chicken	1:200 (ICC)	Jackson ImmunoResearch (West Grove, USA)

ICC – Immunicytochemistry
WB – Western blot

Methods

Rat ghrelin, MK-0677 and [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P Application

Hippocampal organotypic slices (3 or 4 DIV) were treated with the GHS-R1a agonist MK-0677 (1 µM), the GHS-R1a inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P (1 µM) and rat ghrelin (1 µM) for 20 hours or with the agonist MK-0677 in the presence of the GHS-R1a antagonist [D-Lys^3]-GHRP-6 (100 µM), TTX (1 µM) and AP5 (100 µM). 6 DIV hippocampal organotypic slices were treated with the GHS-R1a agonist MK-0677 (1 µM), the GHS-R1a inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P (1 µM) and the GHS-R1a antagonist [D-Lys^3]-GHRP-6 (100 µM) for 20 h. For the biochemistry experiments the antagonist was added 30 min before the agonist. Hippocampal neurons in culture were incubated with the GHS-R1a agonist MK-0677 1 µM for 1 hour (at 19 DIV) or for 20 h (at 18 DIV). When the GHS-R1a antagonist [D-Lys^3]-GHRP-6 (100 µM) was present, it was added 30 min before the agonist. The compounds were all added directly into the culture medium.

Neuronal Culture

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 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4·2H_2O$, 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 [Minimum Essential Medium (MEM; GIBCO, Invitrogen, Barcelone, Spain) supplemented with 10% (v/v) horse serum (GIBCO, Invitrogen, Barcelone, Spain), 0.6% (w/v) glucose, and 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, Barcelone, Spain) supplemented with B27 supplement (1:50 dilution; GIBCO, Invitrogen, Barcelone, Spain), 25 µM glutamate, 0.5 mM glutamine, and 0.12 mg/ml gentamycin (GIBCO, Invitrogen, Barcelone, 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 d. 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. Cultures were used after 7,15, 16, 19 or 21 d.

Slice Culture and Sindbis Virus Expression

Hippocampal slices were prepared from young Wistar rats of either sex (postnatal day 5–6) as previously described (Gähwiler et al., 1997). Briefly, after dissection of the hippocampi in ice cold gassed (5% CO_2/95% O_2) dissection solution (in mM: 10 glucose, 4 KCl, 24 $NaHCO_3$, 234 sucrose, 0.5 $MgCl_2{\cdot}6H_2O$, 0.7 $CaCl_2{\cdot}2H_2O$, 0.03 phenol red, at pH 7.4), 400 µm transverse slices were prepared using a tissue slicer. Slices were transferred to slice culture inserts (Millipore, Madrid, Spain) and cultured in culture medium [Minimum Essential Media (MEM; GIBCO, Invitrogen, Barcelone, Spain) supplemented with 20% (v/v) horse serum (GIBCO, Invitrogen, Barcelone, Spain), 1 mM glutamine, 1 mM $CaCl_2$, 2 $MgSO_4$, 1 mg/l insulin, 0.0012% (w/v) ascorbic acid, 30 mM HEPES, 13 mM glucose, 5.2 mM $NaHCO_3$, at pH 7.25, and final osmolarity of 320 mOsm/l]. Cultures were maintained in a humidified incubator of 5% CO_2/95% air at 35.5°C, and the culture medium was replaced every 2–3 d. The recombinant GluA1-GFP was delivered into slices using Sindbis virus after 1–2 in culture, as previously described (Malinow et al., 2010). Recombinant protein expression was typically for 2 d.

Biochemistry

Protein extracts were prepared in lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100 supplemented with 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml pepstatin (CLAP) and a cocktail of phosphatase inhibitors (1x, Roche, Carnaxide, Portugal)]. After centrifugation at 16,100 x *g* for 10 min at 4ºC, protein in the supernatant was quantified using the bicinchoninic acid (BCA) assay kit (Pierce, Termo Fisher Scientific, Rockford, USA), and the samples were denatured with 5x concentrated denaturating buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) Glicerol, 2% (v/v) SDS, 0.01% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol (added fresh)], and boiled for 5 min. Protein extracts were resolved by SDS-PAGE in 7.5% or 12% polyacrylamide gels. For western blot analysis, proteins were transferred onto a PVDF membrane (Millipore, Madrid, Spain) by electroblotting (40 V, overnight at 4ºC). The membranes were blocked for 1 h at room temperature in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% (v/v) Tween-20 (TBS-T), and 5% (w/v) low-fat milk or BSA. Membranes were probed during 1 h, at room temperature, or overnight, at 4ºC, with the primary antibodies diluted in TBS-T containing 5% or 0.5% (w/v) low-fat milk or 5% (w/v) BSA. Following several washes, membranes were incubated for 1 h with alkaline phosphatase conjugated secondary antibodies (anti-mouse or anti-rabbit, depending on the primary antibody host species) at room temperature, washed again and incubated with chemifluorescent substrate (ECF) (GE Heathcare, Carnaxide, Portugal) for 5 min at room temperature. Membranes were scanned with the Storm 860 scanner (GE Heathcare,

Carnaxide, Portugal), and quantified using the ImageQuant software under linear exposure conditions. When necessary, the membranes were stripped (0.2 M NaOH for 5 min) and re-probed.

Subcellular Fractionation

The procedure for purification of synaptossomes was adapted from Peça et al., 2011. Two hundred mg of hippocampi, dissected from adult Wistar rats, were collected and homogenized in a motor driven glass Teflon homogenizer (30 stokes, 900 rpm, at 4ºC) in HEPES-buffered sucrose (HBS) buffer [0.32 M sucrose, 4 mM HEPES (pH 7.4)] supplemented with protease and phosphatase inhibitors (0.2 mM PMSF, 1 µg/ml CLAP, 0.1 mM Na_3VO_4, and 50 mM NaF). The hippocampal homogenate was collected and centrifuged at 900 x *g* for 15 min, at 4ºC, to obtain the non-nuclear fraction (S1). The resultant supernatant was centrifuged at 18,000 x *g* for 15 min, at 4ºC, to yield the crude synaptossomal pellet (P2). P2 was resuspended in HBS (non-supplemented with Na_3VO_4 and supplemented also with the protease inhibitor 1 mM DTT) and centrifuged at 18,000 x *g* for 15 min, at 4ºC, to yield the washed crude synaptossomal fraction. Protein concentrations were determined using the BCA assay kit (Pierce, Termo Fisher Scientific, Rockford, USA), and samples were denatured with 5x concentrated denaturating buffer, warmed for 5 min at 95ºC, and separated by SDS-PAGE using an equal amount of protein for each fraction.

Neuron Transfection

GFP-tagged GHS-R1a (GHS-R1a-GFP) was recombinantly expressed in primary cultures of hippocampal neurons at 9 DIV using the calcium phosphate transfection protocol (adapted from Jiang et al., 2004). The GHS-R1a-GFP plasmid (4 µg per coverslip) was 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 10 mM 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 1–3 h in the presence of kynurenic acid (2 mM). Each coverslip was transferred to a freshwell of the 12-well plate containing 1 ml 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 10–15 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. Protein expression was typically for 7 d.

Immunocytochemistry

For labeling surface GluA1-containing AMPA receptors, live neurons were incubated for 10 min at room temperature using an antibody against an extracellular epitope in the GluA1 N-terminus diluted in conditioned neuronal culture medium or extracellular solution (used for chemical LTP). Neurons were then fixed for 15 min in 4% sucrose and 4% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 and 10 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.4) at room temperature, and permeabilized with PBS + 0.25% (v/v) Triton X-100 for 5 min, at 4ºC. Neurons were then incubated in 10% (w/v) BSA in PBS for 30 min at 37ºC to block nonspecific staining, and incubated in appropriate primary antibody diluted in 3% (w/v) BSA in PBS (2 h, 37ºC or overnight, 4ºC). After washing 6 times in PBS, cells were incubated with the secondary antibody diluted in 3% (w/v) BSA in PBS (45 min, 37ºC). The coverslips were mounted using fluorescent mounting medium from DAKO (Glostrup, Denmark).

chemical LTP (cLTP) Protocol

cLTP was induced as previously described (Ahmad et al., 2012). 19 DIV hippocampal cultures were thoroughly washed with extracellular solution (ECS) containing (in mM): 150 NaCl, 2 $CaCl_2$, 5 KCl, 10 HEPES, 30 Glucose, 0.001 TTX, 0.01 strychnine, 0.03 picrotoxin, pH 7.4. After washing, neurons were stimulated with or without glycine (300 μM) at room temperature for 3 min in ECS and then incubated for 20–25 min in ECS (no added glycine) in a 37°C, 5% CO_2/95% air incubator. Surface GluA1-AMPAR were labeled, fixed and probed as described above.

Quantitative Imaging Analysis

Imaging was performed on a Zeiss Axiovert 200M microscope and on Zeiss LSM 510 Meta or Zeiss LSM 710 confocal microscopes using a 63 X 1.4 numerical aperture oil objective. Images were quantified using image analysis software (ImageJ). For quantification, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The region of interest was randomly selected avoiding primary dendrites, and dendritic length was measured using MAP2 staining. Measurements were performed in 2–5 independent preparations, and at least 7 cells per condition were analyzed for each preparation.

GHS-R1a and GHS-R1a-GFP

For GHS-R1a (endogenous and exogenous) measurements, the PSD-95 and Vglut1 signals were thresholded and their colocalization was determined. The GHS-R1a signal was measured after thresholds were set so that recognizable clusters were included in the analysis, and GHS-R1a signal present in glutamatergic synapses was obtained by measuring the GHS-R1a puncta positive for both PSD-95 and Vglut1. The number of glutamatergic synapses

containing GHS-R1a per dendritic length was determined by identifying PSD-95- and Vglut1-positive and PSD-95 clusters that were also labeled for GHS-R1a.

GluA1

For quantifying the surface GluA1 signal, digital images were subjected to a user-defined intensity threshold to select clusters and measured for cluster intensity, number, and area for the selected region. The synaptic GluA1 clusters were selected by their overlap with thresholded Vglut1 signal.

Electrophysiology

Voltage-clamp whole-cell recordings were performed stimulating Schaffer collateral fibers and recording evoked synaptic responses from CA1 pyramidal neurons at holding potentials. The AMPA/NMDA ratios were calculated by acquiring AMPAR responses at –60 mV and NMDAR responses at +40 mV at a latency at which AMPAR responses were fully decayed (60 ms after stimulation). Picrotoxin (100 μM) was present in the external solution to block the $GABA_A$R responses. The NMDA/GABA ratios were calculated by recording NMDAR responses at –60 mV and $GABA_A$R responses at 0 mV, in the absence of Mg^{2+}. CNQX (10 μM) was present in the external solution to block AMPAR responses. For the rectification studies, GluA1-GFP was expressed in CA1 neurons for 48 h, and AMPAR responses were recorded at –60 mV and +40 mV in the presence of 100 μM AP5 (external solution) and 100 μM spermine (internal solution). LTP was induced using a pairing protocol by stimulating Schaffer collateral fibers at 3 Hz for 1.5 min (540 pulses) while depolarizing the CA1 postsynaptic cell to 0 mV. Prior to recording, a cut was made between CA3 and CA1 to minimize recurrent activity. The recording chamber was perfused with external solution (in mM: 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 11 glucose, 26 $NaHCO_3$, 4 $MgCl_2$, 4 $CaCl_2$ and 0.004 2-chloroadenosine, at pH 7.4), and was gassed with 5% CO_2/95% O_2. Patch recording pipettes (3–6 M Ω) were filled with internal solution (in mM: 115 $CsMeSO_3$, 20 CsCl, 10 HEPES, 2.5 $MgCl_2$, 4 Na_2ATP, 0.4 Na_3GTP, 10 sodium phosphocreatine and 0.6 EGTA, at pH 7.25). Synaptic responses were evoked with bipolar electrodes using single-voltage pulses (200 μs, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 300 and 500 μm from the CA1 recorded cells. Synaptic responses were averaged over 50 trials. Whole-cell recordings were carried out with a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, USA).

Statistical Analysis

All graphs represent average values ± s.e.m. Statistical differences were calculated according to non-parametric tests for most part of the cases; when parametric tests were used data were first converted to logarithm. Mann-Whitney test or Paired t test 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 test.

Chapter 3

Ghrelin Triggers the Synaptic Incorporation of AMPA Receptors in the Hippocampus

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Submitted for publication

Summary

Ghrelin is a peptide mainly produced by the stomach and released into circulation, affecting energy balance and growth hormone release. These effects are guided largely by the expression of the ghrelin receptor (GHS-R1a) in the hypothalamus and pituitary. However, GHS-R1a is expressed in other brain regions, including in the hippocampus, where its activation enhances memory retention. Herein, we explore the molecular mechanism underlying the hippocampal action of ghrelin. Our data show that GHS-R1a is localized in the vicinity of hippocampal excitatory synapses, and that its activation increases delivery of AMPA receptors (AMPARs) to synapses. These changes are paralleled by functional modifications at excitatory synapses. Moreover, GHS-R1a activation enhances two different paradigms of long-term potentiation in the hippocampus and increases AMPARs and stargazin phosphorylation. These results indicate that GHS-R1a activation enhances excitatory synaptic transmission in the hippocampus by regulating AMPARs trafficking.

Introduction

The appetite stimulating peptide ghrelin is a 28 amino acids peptide predominantly produced by X/A-like cells in the oxyntic glands of the stomach as well as in the intestine (Date et al., 2000a), and secreted into the blood stream. This peptide promotes pituitary growth hormone secretion, through activation of the growth hormone secretagogue type 1a receptor [GHS-R1a or ghrelin receptor (Kojima et al., 1999)]. Additionally, ghrelin is involved in the regulation of energy balance by increasing food intake and reducing fat utilization (reviewed in Gao and Horvath, 2007). Plasma ghrelin levels rise before meals and decrease thereafter (Cummings et al., 2001), a pattern which is consistent with the implication of ghrelin in preprandial hunger and meal initiation. Ghrelin is secreted to the circulation and crosses the blood-brain barrier (Banks et al., 2002; Diano et al., 2006), but there is also evidence for ghrelin synthesis locally in the brain (Kojima et al., 1999; Lu et al., 2002; Cowley et al., 2003). The GHS-R1a receptor mRNA was initially found in the hypothalamus and in the pituitary gland (Howard et al., 1996), and later detected in the hippocampus (Guan et al., 1997). GHS-R1a is a G protein-coupled seven-transmembrane domain receptor, which can signal through $G_{q/11}\alpha$ protein to activate phosphatidylinositol-specific phospholipase C, but which can activate other signaling cascades through various G protein subunits, including $G_q\alpha$ and $G_{i/o}\alpha$ (reviewed in Gao and Horvath, 2007).

The finding that GHS-R1a is expressed in the hippocampus raises the possibility that ghrelin similarly to other appetite-regulating hormones such as leptin (Moult et al., 2010) may affect brain functions other than those related to endocrine and metabolic regulation (reviewed in Andrews, 2011). Indeed, in the last few years several studies have shown that ghrelin increases memory retention in rodents, and that the hippocampus participates in this effect (Carlini et al., 2002, 2004; Diano et al., 2006; Chen et al., 2011). Ghrelin deficient mice exhibit decreased novel object recognition, a type of memory test dependent on hippocampal function (Diano et al., 2006), suggesting that endogenous ghrelin has a physiological role in improving learning and memory. Additionally, 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 (Wu et al., 2003; Stranahan et al., 2008). On the other hand, 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) while increasing learning consolidation and facilitating synaptic plasticity (Fontán-Lozano et al., 2007). Recent evidence suggests an enhancing effect of ghrelin on long-term potentiation (LTP) in the hippocampus (Diano et al., 2006; Chen et al., 2011), a form of activity-dependent synaptic plasticity which is the cellular correlate for learning and memory (Bliss and Collingridge, 1993). However, one study did not observe effects of ghrelin on LTP induced by theta burst stimulation (Diano et al., 2006), while the other only detected effects of ghrelin on a late phase of LTP (2h after high-frequency stimulation; Chen et al., 2011).

Although the function of ghrelin as a cognitive enhancer is well documented, the molecular mechanisms that underlie this function are still poorly understood. Here, we have tested whether the activation of GHS-R1a affects the trafficking of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs), crucial for the expression of changes in synaptic strength in the hippocampus (reviewed in Santos et al., 2009). We report that GHS-R1a localizes to excitatory synapses and that its activation induces the synaptic delivery of GluA1-AMPAR in rat hippocampal cultures and in CA1 cells in organotypic hippocampal slices. These changes enhance excitatory synaptic transmission. Furthermore, we show that ghrelin receptor activation enhances LTP expression in the CA3-CA1 synapse in organotypic hippocampal slices, and increases the synaptic and cell surface trafficking of GluA1-AMPAR induced by chemical LTP in hippocampal cultures. Finally, we demonstrate that ghrelin receptor activation in the hippocampus increases the phosphorylation of GluA1 and stargazin. Taken together our data indicate that ghrelin receptor activation regulates AMPARs trafficking underlying synaptic plasticity and learning.

Results

GHS-R1a Localizes to Excitatory Hippocampal Synapses

Previous evidence suggests that GHS-R1a is expressed in the hippocampus (Diano et al., 2006; Cuellar and Isokawa, 2011), but the subcellular localization of GHS-R1a in hippocampal cells has not been studied. Primary hippocampal neurons were transfected with GFP-tagged GHS-R1a (Leung et al., 2007) and immunolabeled at 16 DIV for GFP. GHS-R1a-GFP is distributed throughout dendrites and forms clusters that partially colocalize with the glutamatergic synapse markers PDS-95, a postsynaptic scaffold, and Vglut1, a presynaptic vesicular glutamate transporter (Figure 1A). To evaluate the presence of GHS-R1a-GFP at excitatory synapses, we identified regions of overlap between the PSD-95 and Vglut1 signals and measured the GHS-R1a-GFP immunolabeling at these sites (Figure 1B). We found that 37.9±0.05% of the clusters positive for both PSD-95 and Vglut1, presumably corresponding to functional synapses, contain GHS-R1a-GFP (Figure 1B and 1C), which accounts for 47.3±4.6% of the total GHS-R1a-GFP fluorescence intensity in dendrites. Interestingly, only 20.6±0.01% of the PSD-95 positive sites contain GHS-R1a-GFP (Figure 1C), suggesting that GHS-R1a is preferentially localized to the vicinity of functional synapses. This distribution pattern was confirmed using an antibody against the endogenous GHS-R1a (Figures S1A–S1C). Furthermore, we observed that GHS-R1a is enriched in purified crude synaptosomes (P2 fraction) from adult rat hippocampi (Figure 1D), consistent with a synaptic expression of GHS-R1a in the adult rat hippocampus. Finally, we found that GHS-R1a expression levels significantly increase in cultured hippocampal neurons from 7 DIV up to 19 DIV (Figure S1D). Taken together, this evidence indicates that a significant fraction of GHS-R1a is localized in the vicinity of hippocampal glutamatergic synapses, and that its expression is regulated during development, thus suggesting a role for ghrelin in regulating excitatory synaptic transmission.

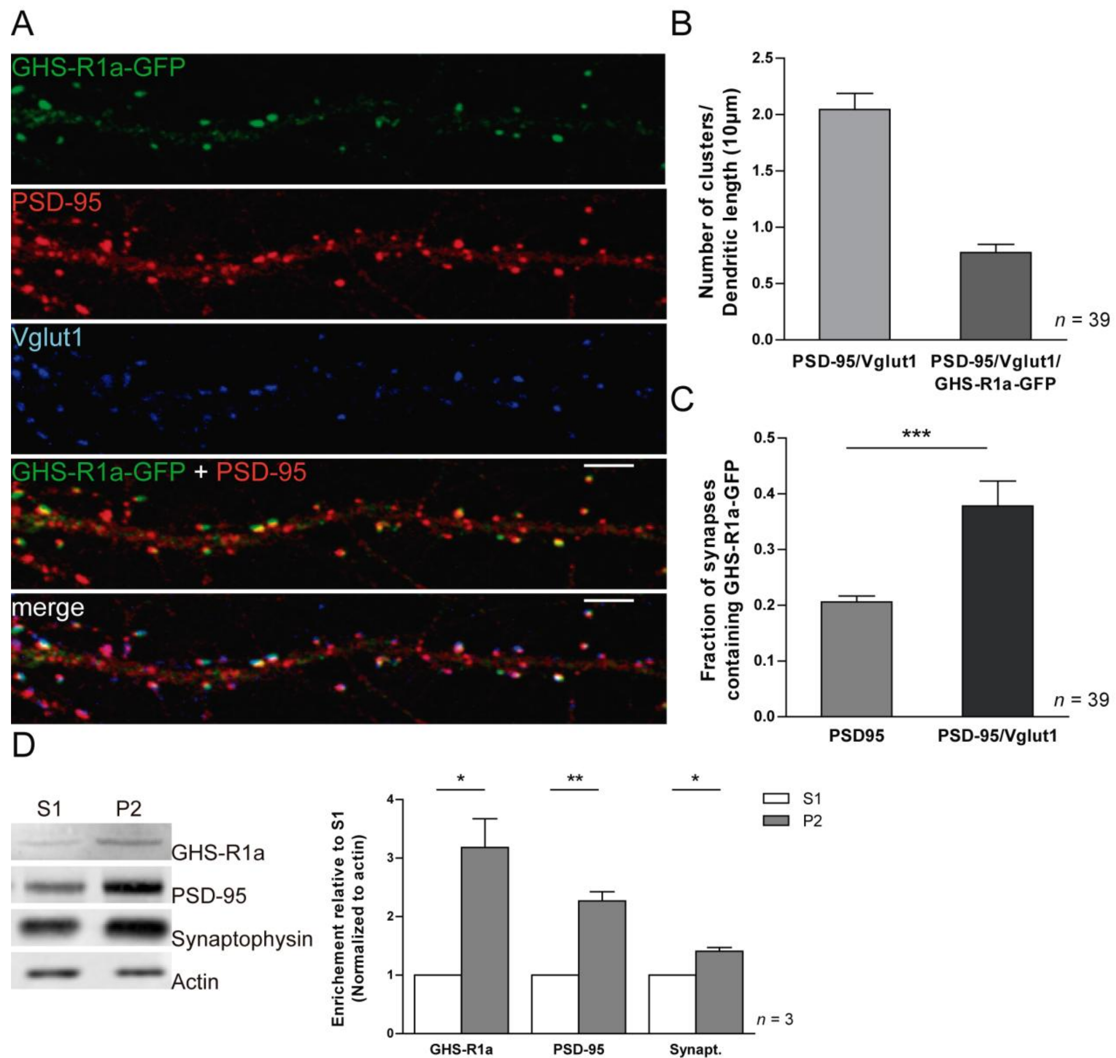

Figure 1. GHS-R1a localizes to excitatory synapses in the hippocampus. (**A**) Representative immunofluorescence images of hippocampal neurons in culture transfected with a GFP-GHS-R1a construct. Neurons were stained for PSD-95 and Vglut1. Scale bars represent 5 µm. (**B**) Quantification of the number of dendritic clusters per dendritic length that are positive for both PSD-95 and Vglut1 (functional synapses) and of the number of glutamatergic synapses that contain GHS-R1a-GFP (PSD-95/Vglut1/GHS-R1a-GFP). *n* represents the total number of analyzed cells in three independent experiments. Error bars represent s.e.m. (**C**) GHS-R1a-GFP is preferentially localized to the vicinity of functional excitatory sinapses. The fraction of synapses containing GHS-R1a-GFP was calculated by evaluating the presence of GHS-R1a-GFP at regions of overlap between PSD-95 and Vglut1 clusters, or at PSD-95 clusters. *n* represents the total number of analyzed cells in three independent experiments. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test (***$P < 0.001$). (**D**) GHS-R1a is enriched in purified crude synaptosomes. Synaptosomal fractions (P2) isolated from adult rat hippocampi were analyzed for the presence of GHS-R1a, PSD-95, synaptophysin (Synapt.) and actin, as indicated. The plot indicates the enrichment in each protein in the synaptosomal fraction relatively to hippocampal homogenate fraction (S1), normalized to actin. Error bars represent s.e.m. The statistical significance was calculated using the Paired t test (*$P < 0.05$ and **$P < 0.01$). *n* represents the number of independent experiments.

GHS-R1a Activation Enhances Excitatory Synaptic Transmission by Inducing GluA1-AMPAR Synaptic Delivery

To explore the effect of ghrelin receptor activation on the cell surface and synaptic expression of GluA1-AMPAR, we performed quantitative immunofluorescence analysis of the expression of synaptic cell surface GluA1 in hippocampal neurons treated with the GHS-R1a agonist MK-0677. Hippocampal neurons were treated with the GHS-R1a agonist (1 μM, 1 h), or with the agonist simultaneously with the GHS-R1a antagonist [D-Lys^{3}]-GHRP-6 (100 μM), and live-stained with an antibody against an extracellular epitope in the GluA1 N-terminus. After fixation, neurons were stained for MAP2 to visualize the dendritic structure, and for PSD-95 and Vglut1 to visualize excitatory synapses. We verified that the treatment with the agonist significantly increase the total fluorescence intensity of PSD-95 (Figure S2A), which was blocked in the presence of the GHS-R1a antagonist (Figure S2A), and did not have any effect on the total fluorescence intensity of Vglut1 (Figure S2B). Thus, suggesting an increase in dendritic spine density upon ghrelin receptor activation, as described before (Diano et al., 2006; Berrout and Isokawa, 2012). Considering the previous finding the excitatory synaptic marker Vglut1 was used to define the cell surface synaptic GluA1 clusters. Neurons treated with the agonist showed a significant increase in the fluorescence intensity of total and Vglut1-colocalized GluA1 surface clusters (Figures 2A–2C), which was abolished in the presence of the GHS-R1a antagonist (Figures 2A–2C). These observations suggest that MK-0677 increases the levels of AMPARs at the neuronal surface and at synapses through a mechanism that is specifically mediated by the ghrelin receptor.

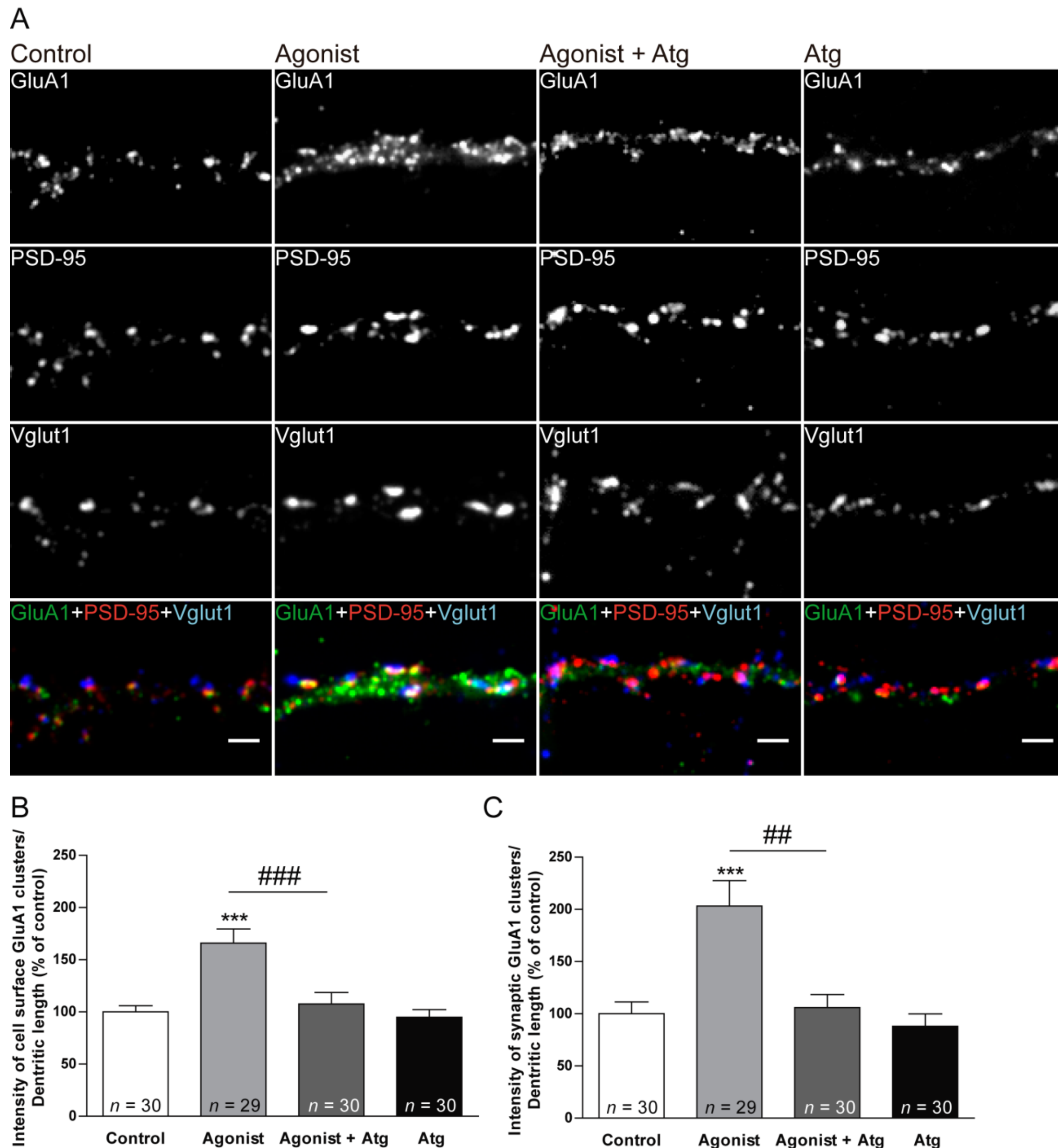

Figure 2. Ghrelin receptor activation promotes the synaptic expression of GluA1. (**A**) Hippocampal neurons 19 DIV were incubated with the ghrelin receptor agonist MK-0677 (1 μM) for 1 h, with the agonist in the presence of the ghrelin receptor antagonist [D-Lys^{3}]-GHRP-6 (Atg, 100 μM), or with the antagonist (Atg) alone. Neurons were live stained for GluA1 using an antibody against an extracellular epitope in the GluA1 N-terminus. After fixation, neurons were stained for PSD-95, Vglut1 and MAP2. Scale bars represent 2 μm. Neurons were analyzed for the total GluA1 cell surface fluorescence intensity (**B**) and for the GluA1 synaptic cluster (Vglut1-colocalized) fluorescence intensity (**C**) per dendritic length. (**B**,**C**) Results are expressed as % of control cells, and are averaged from three independent experiments. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test (***$P < 0.001$). Comparisons between pairs were performed using the Mann-Whitney test ($^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$). *n* represents the total number of analysed cells in independent preparations.

To directly determine whether GHS-R1a activation induces the delivery of new AMPARs into synapses, we expressed GluA1-GFP in CA1 neurons in organotypic hippocampal slice

cultures. Overexpression of GluA1, with a Sindbis virus expression system, leads to the formation of homomeric AMPARs containing the GluA1 subunit (Hayashi et al., 2000). These GluA2-lacking receptors are inwardly rectifying (Boulter et al., 1990; Hollmann et al., 1991; Verdoorn et al., 1991), and therefore their recruitment to the synapse can be monitored as an increase in the ratio of the evoked post synaptic current at –60 mV relative to the current at +40 mV [rectification index; (Hayashi et al., 2000)]. In this system it was previously shown that newly synthesized GluA1-containing AMPARs are not spontaneously inserted at synapses, unless driven by strong synaptic stimulation or activation of specific signaling pathways associated with LTP induction (Hayashi et al., 2000; Boehm et al., 2006). Organotypic slice cultures were infected with GluA1-GFP at 1 DIV or 2 DIV, and the electrophysiology recordings were performed at 3 or 4 DIV. Treatment of organotypic slices with ghrelin (1 μM, Figure 3A) or with the GHS-R1a agonist MK-0677 (1 μM, Figure 3B) for 20 h did not change the rectification index in uninfected cells, indicating that GHS-R1a activation does not alter the intrinsic rectification properties of endogenous AMPARs. Interestingly, we found that slice treatment with either ghrelin (Figure 3A) or MK-0677 (Figure 3B) increases the rectification index in neurons that express GluA1-GFP, strongly suggesting that ghrelin receptor activation induces synaptic delivery of AMPARs. Importantly, the effect of MK-0677 on the synaptic delivery of GluA1-AMPAR was blocked by the GHS-R1a antagonist [D-Lys^3]-GHRP-6 (100 μM, Figure 3B). In addition, we tested whether the observed effect requires synaptic activity, and found that in the presence of the NMDAR antagonist AP5 (100 μM), or of the voltage-gated sodium channels blocker tetrodotoxin (TTX, 1 μM), the rectification index of GluA1-GFP-expressing neurons was not significantly changed upon incubation with the GHS-R1a agonist MK-0677 (Figure 3B). Taken together these results indicate that activation of the hippocampal ghrelin receptor induces delivery of GluA1 homomeric receptors into synapses in an activity-dependent manner.

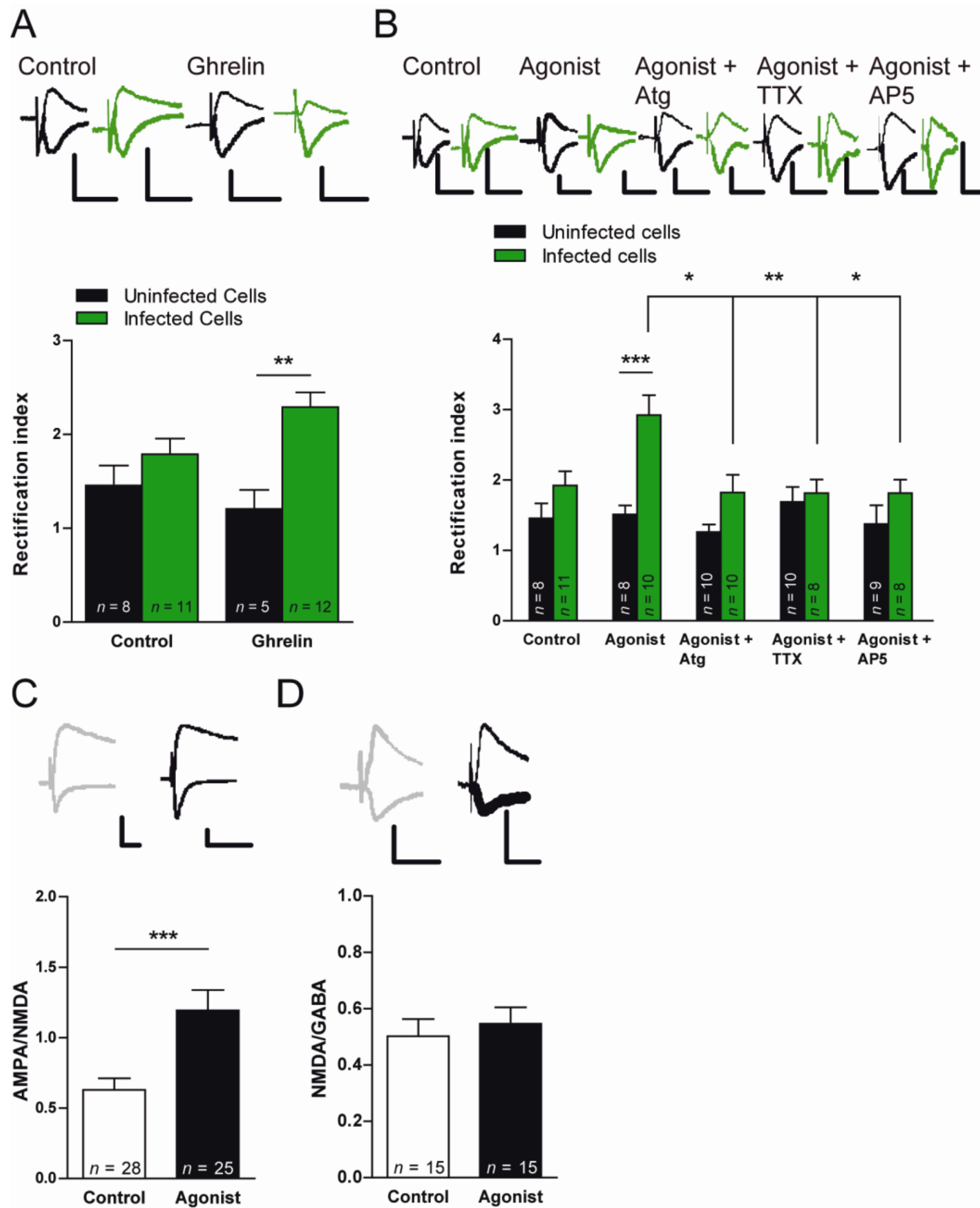

Figure 3. Ghrelin receptor activation induces activity-dependent GluA1-AMPAR synaptic delivery in CA1 neurons. (**A**,**B**) Voltage-clamp whole cell recordings obtained in CA1 neurons expressing GluA1-GFP (Infected cells) and adjacent non-fluorescent neurons (Uninfected cells). GluA1 synaptic delivery was quantified as an increase in rectification index (RI = I_{-60}/I_{+40}). (**A**) Ghrelin induces GluA1-AMPAR synaptic delivery in CA1 neurons. Average RIs for ghrelin-treated cells (1 µM, 20 h) and untreated cells. (**B**) The ghrelin receptor agonist MK-0677 induces activity-dependent GluA1-AMPAR synaptic delivery. Average RIs for untreated cells, ghrelin receptor agonist-treated cells (1 µM, 20 h), ghrelin receptor agonist-treated cells in the presence of the antagonist of the ghrelin receptor [D-Lys^3]-GHRP-6 (Atg, 100 µM), the voltage-gated sodium channels inhibitor tetrodotoxin (TTX) or the NMDAR antagonist AP5. (**C**,**D**) Comparison of evoked synaptic responses in control organotypic hippocampal slices and in slices treated for 20 h with the ghrelin receptor agonist MK-0677 (1 µM). (**C**) Average AMPA/NMDA ratios for agonist-treated and control cells. (**D**) Average NMDA/GABA ratios for agonist-treated and control cells. (**A**–**D**) Representative traces appear above the corresponding bars. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test (*P < 0.05, **P < 0.01 and ***P < 0.001). *n* represents the number of cells. Scale bars: vertical, 50 pA; horizontal, 20 ms.

To evaluate whether the induction of AMPAR synaptic delivery mediated by GHS-R1a activation produces a functional modification at excitatory CA3-CA1 synapses, 2 or 3 DIV organotypic hippocampal slices were treated with the GHS-R1a agonist MK-0677 (1 μM, 20 h) and electrophysiological recordings were performed at 3 or 4 DIV. The AMPA/NMDA ratio of synaptic responses significantly increased after MK-0677 treatment compared with control neurons (Figure 3C), whereas the NMDA/GABA ratio was not changed (Figure 3D). Additionally, MK-0677 treatment did not change the passive membrane properties of the neuron, indicating that cell-wide ion channel conductance was not altered (Figure S2C). These results suggest that GHS-R1a activation produces a functional change at excitatory CA1 synapses, specifically an increase in AMPARs-mediated synaptic transmission. In conclusion, this group of results suggests that GHS-R1a activation enhances excitatory synaptic transmission by inducing the insertion of new AMPARs at synapses.

GHS-R1a Activation Enhances Hippocampal LTP

The classic paradigm for activity-dependent synaptic delivery of AMPARs is NMDAR-dependent LTP. Since our data suggest that the synaptic incorporation of AMPARs induced by GHS-R1a activation is an activity-dependent process we hypothesized that GHS-R1a activation may facilitate LTP-like events in the hippocampus. Therefore, we tested whether ghrelin receptor activation affects this form of synaptic plasticity. Hippocampal organotypic slices (2 or 3 DIV) were treated with the GHS-R1a agonist MK-0677 (1 μM, 20 h) and LTP was induced in the CA3-CA1 synapse. LTP induction significantly increased AMPAR-mediated responses in both MK-0677-treated and untreated neurons (Figure 4A and 4B). However, MK-0677 application dramatically enhanced LTP expression [5.0±0.9 fold potentiation with MK-0677 *vs*. 2.3±0.3 fold potentiation in control neurons (Figure 4A)]. Additionally, MK-0677 treatment did not have an effect on the non-potentiated (unpaired) pathway (Figure S3A). This suggests that GHS-R1a activation facilitates classic NMDAR-dependent LTP.

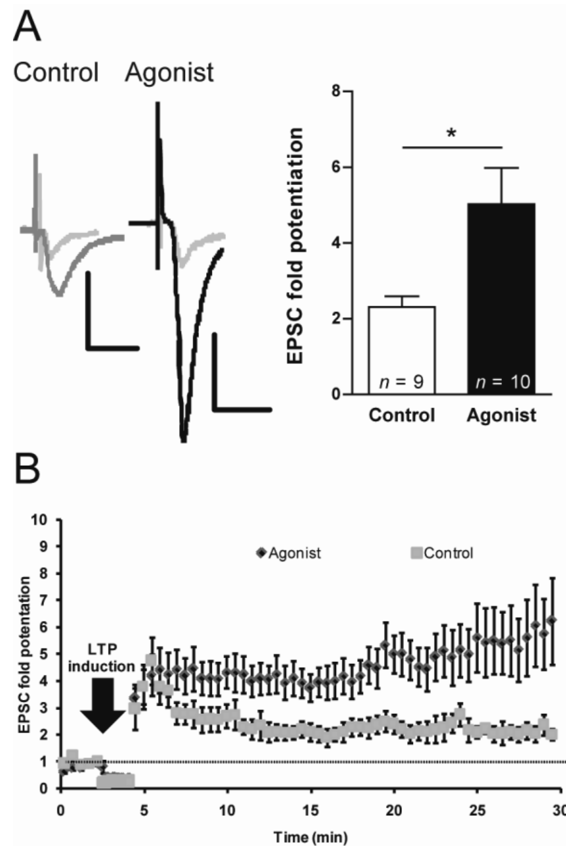

Figure 4. The ghrelin receptor agonist MK-0677 enhances long-term synaptic potentiation in the CA3-CA1 synapse. (**A**) Sample traces of evoked AMPAR-mediated synaptic responses recorded from CA1 neurons at –60 mV before (light gray line) and after (darker line) LTP induction. Scale bars: vertical, 50 pA; horizontal, 20 ms. Organotypic slice cultures were incubated in culture medium, or in medium containing the ghrelin receptor agonist MK-0677 (1 μM, 20 h). Plot shows quantification of average synaptic potentiation from paired pathway 20–30 min after LTP induction. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test (*P < 0.05). *n* represents the number of cells. (**B**) Time course of normalized AMPAR-mediated synaptic responses before and after LTP induction. For simplicity, each time point in the plot corresponds to the average of 12 consecutive stimulations (sampling rate: 0.2 Hz).

To directly test whether GHS-R1a activation affects NMDAR-triggered delivery of endogenous GluA1-containing AMPARs to synaptic sites, we used a neuronal culture model of LTP (chemical LTP, cLTP), in which pharmacological activation of NMDARs leads to an increase in the surface expression of AMPARs (Lu et al., 2001; Passafaro et al., 2001; Park et al., 2004; Kennedy et al., 2010; Ahmad et al., 2012). Consistent with previous results cLTP induction caused a significant increase in the fluorescence intensity of total (Figures 5A and 5B) and Vglut1-colocalized GluA1 surface clusters (Figures 5A and 5C). We found that pre-treatment with the GHS-R1a agonist MK-0677 (1 μM) for 1 h before the cLTP protocol significantly increases the fluorescence intensity of total (Figures 5A and 5B) and Vglut1-colocalized GluA1 surface clusters (Figures 5A and 5C) compared to neurons subjected to cLTP only, suggesting that GHS-R1a activation increases the cLTP-induced delivery of endogenous GluA1-AMPAR to synaptic sites. The same effect was observed for neurons treated for 20 h with MK-0677 (Figures S3B–S3D). The MK-0677-induced increase in the

surface expression of AMPARs upon cLTP was abolished by application of the GHS-R1a antagonist [D-Lys^3]-GHRP-6 (Figures 5A-5C and S3B–S3D), in agreement with an effect specifically mediated by ghrelin receptor activation. These data suggest that the pre-treatment with the agonist produces an alteration in the trafficking of AMPARs, which facilitates their recruitment to the synapse upon cLTP induction. Altogether, these data suggest that GHS-R1a activation in the hippocampus increases the AMPARs delivery to synapses, facilitating the expression of LTP-like events.

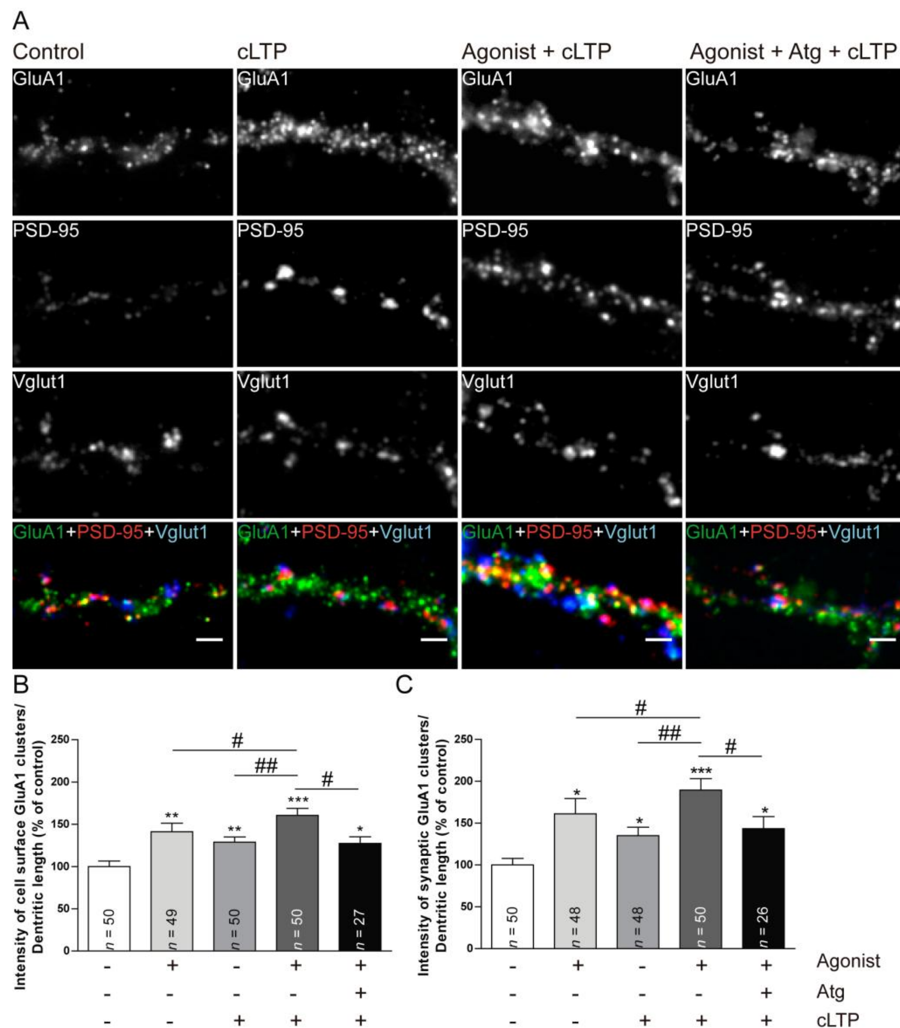

Figure 5. Treatment with the ghrelin receptor agonist MK-0677 increases the cell surface trafficking of GluA1-AMPAR mediated by cLTP. (**A**) Hippocampal neurons in culture were submitted to the following stimuli: cLTP (300 μM glycine for 3 min in the absence of Mg^{2+}), cLTP in neurons treated with the ghrelin receptor agonist MK-0677 (1 μM, 1 h) or simultaneously with the ghrelin receptor agonist and antagonist [D-Lys^3]-GHRP-6 (Atg, 100 μM). Neurons were kept at 37ºC for 20 min (without glycine) and were live stained for surface GluA1. After fixation, neurons were stained for PSD-95, Vglut1 and MAP2. Scale bars represent 2 μm. Neurons were analyzed for the total GluA1 cell surface fluorescence intensity (**B**), and for the GluA1 synaptic cluster (Vglut1-colocalized) fluorescence intensity (**C**) per dendritic length. (**B**,**C**) Results are expressed as % of control cells, and are averaged from 3–5 independent experiments. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test (*P < 0.05, **P < 0.01 and ***P < 0.001). Comparisons between pairs were performed using the Mann-Whitney test ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$). *n* represents the number of cells.

GHS-R1a Activation Increases GluA1 and Stargazin Phosphorylation

Two distinct but interrelated mechanisms regulate AMPAR function: modulation of the receptor ion channel properties (e.g., Derkach et al., 1999) and regulation of the synaptic targeting of the receptor (e.g., Hayashi et al., 2000). Both of these processes are regulated by receptor phosphorylation (reviewed in Henley et al., 2011). We evaluated four key events linked to ghrelin receptor activation and playing an important role in the trafficking of AMPARs and consequently in LTP: PKC activation, PI3 kinase activation, GluA1 phosphorylation and stargazin phosphorylation. These biochemical changes were evaluated in whole-cell extracts from 4 DIV organotypic hippocampal slices at different times after adding the GHS-R1a agonist MK-0677 (1 µM). To evaluate global PKC activation, we monitored the phosphorylation of multiple PKC substrates. MK-0677 treatment led to an upregulation of the PKC pathway, with a significant increase after 5 h of treatment, which was abolished by the GHS-R1a antagonist (Figure S4A). The activation of the PI3 kinase pathway was monitored evaluating the phosphorylation of the PI3 kinase downstream effector Akt at Ser^{473}. As shown in Supplementary Figure 4B, MK-0677 application led to rapid (30 min) significant activation of the PI3 kinase pathway. Phosphorylation of GluA1 at Ser^{831} and Ser^{845}, and stargazin at $Ser^{239/240}$ were evaluated with the corresponding phospho-specific antibodies. GluA1 phosphorylation at Ser^{831} (a PKC substrate, Figure 6A) and GluA1 phosphorylation at Ser^{845} (a PKA substrate, Figure 6B) significantly increased after prolonged treatment with MK-0677. Finally, stargazin phosphorylation at $Ser^{239/240}$ was significantly induced 20 h after the addition of MK-0677 (Figure 6C), an effect which was abolished by the GHS-R1a antagonist. Collectively, these results strongly suggest that ghrelin receptor activation initiates signaling mechanisms ultimately producing post-translational modifications in GluA1-AMPAR and in the AMPAR-associated protein stargazin. These mechanisms may be related to an enhancement of excitatory synaptic transmission, as a consequence of GluA1 synaptic delivery.

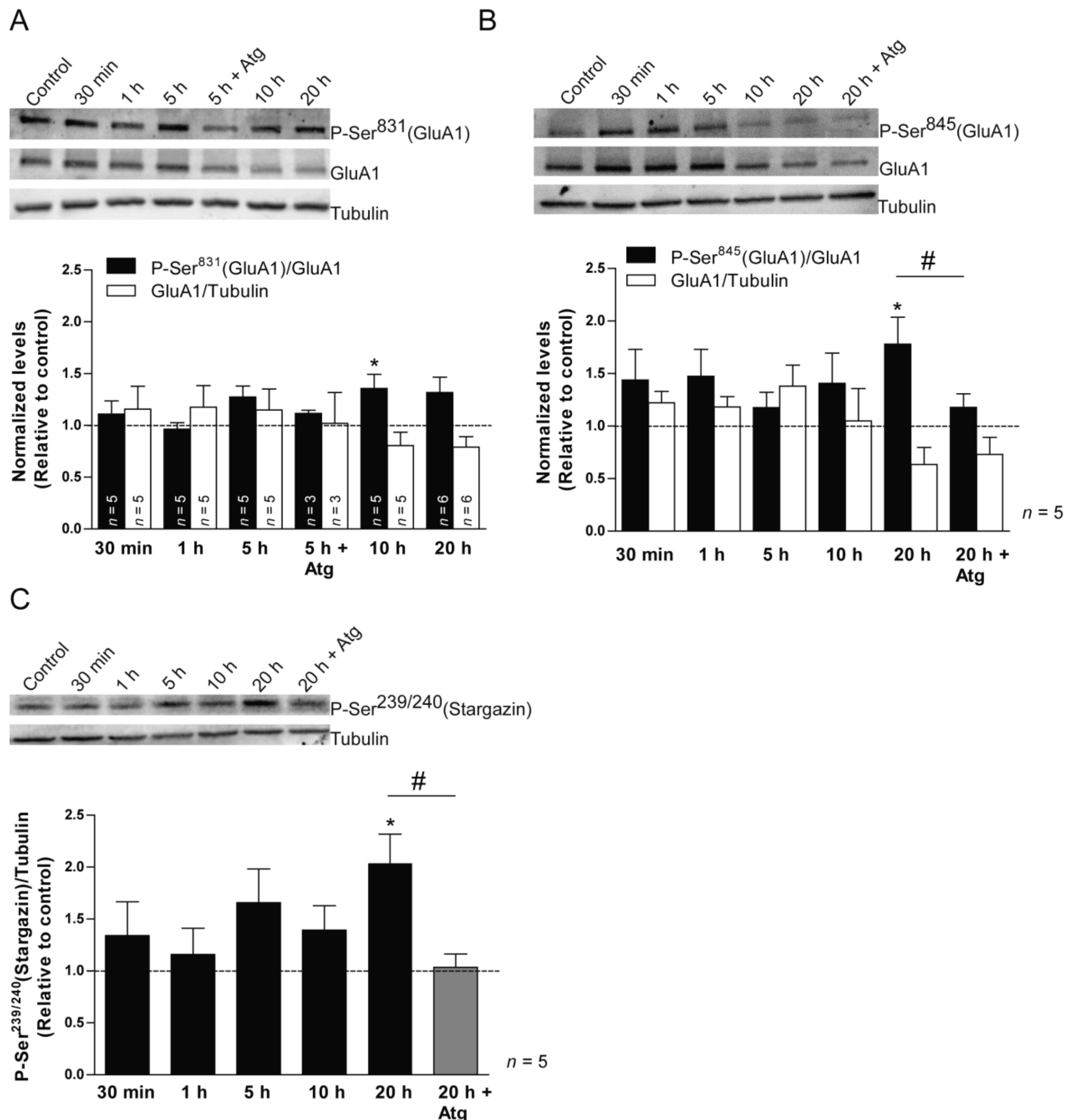

Figure 6. Ghrelin receptor activation triggers changes in GluA1 and stargazin phosphorylation in the hippocampus. (**A**–**C**) Western blot analysis of protein extracts from hippocampal slices incubated with culture medium or with medium containing the ghrelin receptor agonist MK-0677 (1 μM) for the indicated periods of time, or with MK-0677 in the presence of the ghrelin receptor antagonist [D-Lys^3]-GHRP-6 (Atg, 100 μM). The primary antibodies detected phosphorylated GluA1 at Ser^{831} (**A**) and at Ser^{845} (**B**), and phosphorylated stargazin at Ser$^{239/240}$ (**C**). Tubulin was used as a loading control. The graphs represent the quantification of band intensities relative to control extracts. Error bars represent s.e.m. The statistical significance was calculated using the Paired t test (*P < 0.05 and $^{\#}$P < 0.05). *n* represents the number of independent experiments.

Discussion

The localization of the ghrelin receptor in the hippocampus raises the possibility that signaling through this receptor may control higher brain functions, and represent a molecular link between energy metabolism and learning capabilities. A decade ago Carlini and colleagues first suggested that ghrelin could affect cognition (Carlini et al., 2002). In this study, intracerebroventricular injection of ghrelin in rats was found to increase the latency time in the step-down inhibitory avoidance test, suggesting an increase in memory retention. Diano and colleagues showed that ghrelin can enter the CNS and bind to hippocampal neurons, promoting dendritic spine formation. Additionally, they found that ghrelin treatment of acute hippocampal slices enhances the rise in EPSP slope in the CA1 region after a 10 Hz stimulation (Diano et al., 2006). A recent study found that a single ghrelin infusion prolongs the expression of LTP in the dentate gyrus *in vivo* (Chen et al., 2011). Ghrelin knockout animals showed decreased number of spine synapses in the hippocampus and impaired performance in novel object recognition tests, both of which were rapidly reversed by ghrelin administration (Diano et al., 2006). This evidence indicates a function for endogenous ghrelin in modulating hippocampal spines and hippocampal-dependent memory, which is further supported by the fact that ghrelin receptor knockout mice display impairments in the Morris water maze test (Davis et al., 2011). Taken together these studies strongly support a cognition enhancer effect for ghrelin through its hippocampal action. Ghrelin mediated-signaling could provide the link between metabolic requirements, feeding and memory retention, facilitating the successful search for food sources, allowing animals to remember food locations and to retain the successful approach that was used to find them (reviewed in Moran and Gao, 2006 and Olszewski et al., 2008).

Despite all the studies implicating ghrelin and ghrelin receptor mediated-signaling in hippocampal memory processes, little is known about the cellular and molecular mechanisms underlying these effects. In the present study we provide evidence that supports an effect of ghrelin receptor activation on AMPARs synaptic traffic, broadly accepted as a mechanism for the expression of the synaptic plasticity processes thought to be the cellular correlates of learning and memory (reviewed in Collingridge et al., 2004). We found that the ghrelin receptor partially colocalizes with synaptic proteins of glutamatergic synapses in primary hippocampal neurons, and is enriched in synaptosomes purified from adult rat hippocampus, suggesting that it is appropriately localized to modulate excitatory transmission. We report that activation of hippocampal ghrelin receptors promotes the synaptic insertion of AMPARs in the hippocampus. Ghrelin receptor activation resulted in an activity-dependent increase in the inward rectification of AMPAR-mediated responses in CA1 neurons that express GluA1-GFP in hippocampal slices, and produced an increase in the synaptic levels of AMPA receptors in cultured hippocampal neurons. These synaptic changes in AMPARs trafficking were paralleled by an increase in the AMPA/NMDA ratio of synaptic responses from CA1 neurons upon ghrelin receptor activation in hippocampal slices. We also show that activation of the ghrelin receptor dramatically enhances

LTP expression in organotypic hippocampal slices, and increases the synaptic accumulation of GluA1 triggered by cLTP in cultured hippocampal neurons. Altogether our data indicate that ghrelin receptor activation in the hippocampus, besides promoting dendritic spine formation as previously shown (Diano et al., 2006), enhances synaptic plasticity by delivering AMPARs into synapses.

How does ghrelin receptor activation affect AMPA receptor traffic? We found that activation of the ghrelin receptor in the hippocampus leads to changes in the phosphorylation of AMPA receptors and stargazin. Ghrelin receptor activity triggers the phosphorylation of two known PKC substrates, Ser^{831} in GluA1 (Roche et al., 1996) and stargazin (Tomita et al., 2005b). These are also substrates for Ca^{2+}- and calmodulin-dependent protein kinase II (CaMKII) (Mammen et al., 1997; Tomita et al., 2005b), but we could not detect activation of CaMKII upon ghrelin receptor activation (Figure S4C). Phosphorylation of Ser^{831} in GluA1 increases significantly during LTP (e.g., Boehm et al., 2006), but does not seem to be required for receptor synaptic delivery (Hayashi et al., 2000). However, it is possible that other PKC phosphorylation sites in GluA1, such as Ser^{818} (Boehm et al., 2006) and Ser^{816} (Lin et al., 2009), which affect the synaptic incorporation of AMPA receptors, may be phosphorylated upon ghrelin receptor activation. In addition we found that phosphorylation of Ser^{845} in GluA1, a substrate for protein kinase A (Roche et al., 1996) is increased in slices treated with MK-0677, in agreement with a previous study showing that ghrelin can activate PKA in the hippocampus (Cuellar and Isokawa, 2011). Phosphorylation of Ser^{845} in GluA1 is necessary but not sufficient for synaptic incorporation of GluA1-containing AMPARs (Esteban et al., 2003), and was shown to prime AMPARs for synaptic delivery by trafficking them to extrasynaptic sites; subsequent synaptic incorporation requires synaptic NMDARs activation (Oh et al., 2006). In fact, we observed that the increase in AMPARs synaptic delivery mediated by ghrelin receptor activation is blocked by AP5, suggesting a NMDARs-dependent mechanism. The AMPA receptor-associated protein stargazin was also phosphorylated upon ghrelin receptor activation in hippocampal slices. Phosphorylation of stargazin promotes synaptic trafficking of AMPARs, and is required for LTP (Tomita et al., 2005b). Upon phosphorylation stargazin binds to PDZ domain-containing proteins such as PSD-95 and stops the diffusion of cell surface AMPA receptors at synaptic sites (Opazo et al., 2010). Together these results suggest that ghrelin receptor activation induces the synaptic delivery of GluA1-containg AMPARs by upregulating the number of AMPARs that are available for synaptic incorporation, via PKA activity and phosphorylation of GluA1 at Ser^{845}. Ghrelin-induced stargazin phosphorylation will ultimately lead to receptor trapping at the synapse. Additionally we found that short-term treatment with the ghrelin receptor agonist activates the PI3 kinase signaling pathway, in agreement with a previous study in the dentate gyrus (Chen et al., 2011). PI3 kinase has been implicated in the induction (Opazo et al., 2003) and expression (Sanna et al., 2002) of LTP in the CA1 hippocampal region, and was found to be required for AMPA receptor insertion during LTP (Man et al., 2003). PI3 kinase is responsible for a constant supply of PIP3 necessary to ensure PSD-95-mediated clustering of

AMPARs at the postsynaptic membrane (Arendt et al., 2010). Interestingly, the biochemical changes triggered by ghrelin in the hippocampus do not occlude further LTP expression. In fact, LTP expression in organotypic hippocampal slices and delivery of GluA1-containing AMPARs induced by chemical LTP in hippocampal cultured neurons are both enhanced upon ghrelin receptor activation. We thus propose that ghrelin receptor activation-triggered signaling acts on targets that facilitate LTP expression. In conclusion, the present study has provided mechanistic insights into the synaptic events and molecular cascades that mediate enhanced cognition produced by ghrelin.

In addition to inducing a robust feeding response through its hypothalamic action, ghrelin affects other feeding-related behaviors including olfactory function (Tong et al., 2011), motivational aspects of feeding (Abizaid et al., 2006; Malik et al., 2008), and hippocampal memory retention (Carlini et al., 2002, 2004; Diano et al., 2006; Atcha et al., 2009; Chen et al., 2011; Davis et al., 2011). Similarly to what we describe here for the hippocampal action of ghrelin, it is possible that the effect of ghrelin on other brain regions involves the modulation of glutamatergic transmission.

Supplementary Figures

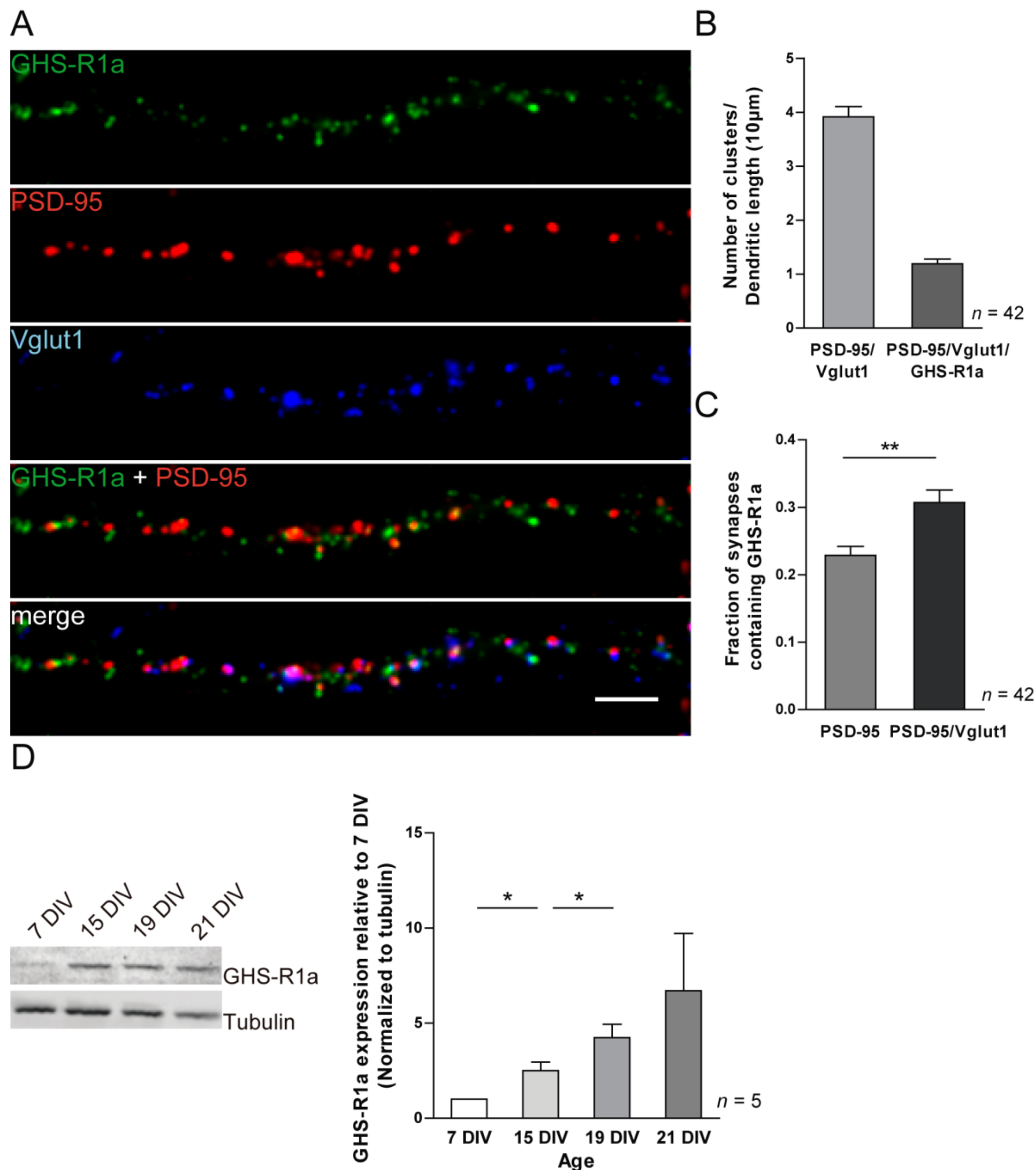

Fig. S1. GHS-R1a localizes preferentially to functional synapses and is developmentally regulated in the hippocampus. (**A**) Representative immunofluorescence images of 15 DIV hippocampal neurons in culture stained for GHS-R1a, PSD-95 and Vglut1.Scale bars represent 5 µm. (**B**) Quantification of the number of clusters per dendritic length that are positive for both PSD-95 and Vglut1 and of the number of glutamatergic synapses that contain GHS-R1a (PSD-95/Vglut1/GHS-R1a). *n* represents the total number of analyzed cells in four independent experiments. Error bars represent s.e.m. (**C**) The endogenous GHS-R1a localizes preferentially to the vicinity of functional synapses. The fraction of synapses containing GHS-R1a was calculated by evaluating the presence of GHS-R1a at regions of overlap between PSD-95 and Vglut1 clusters, or at PSD-95 clusters. *n* represents the total number of analyzed cells in four independent experiments. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test (**P < 0.01). (**D**) Developmental profile for the expression of GHS-R1a in hippocampal cultured neurons. GHS-R1a expression levels increase from 7 DIV to 19 DIV, remaining high up to 21 DIV. Tubulin was used as a loading control. The plot represents the mean intensity of GHS-R1a bands normalized to tubulin, relative to 7 DIV. Error bars represent s.e.m. The statistical significance was calculated using the Paired t test (*P < 0.05). *n* represents the number of independent experiments.

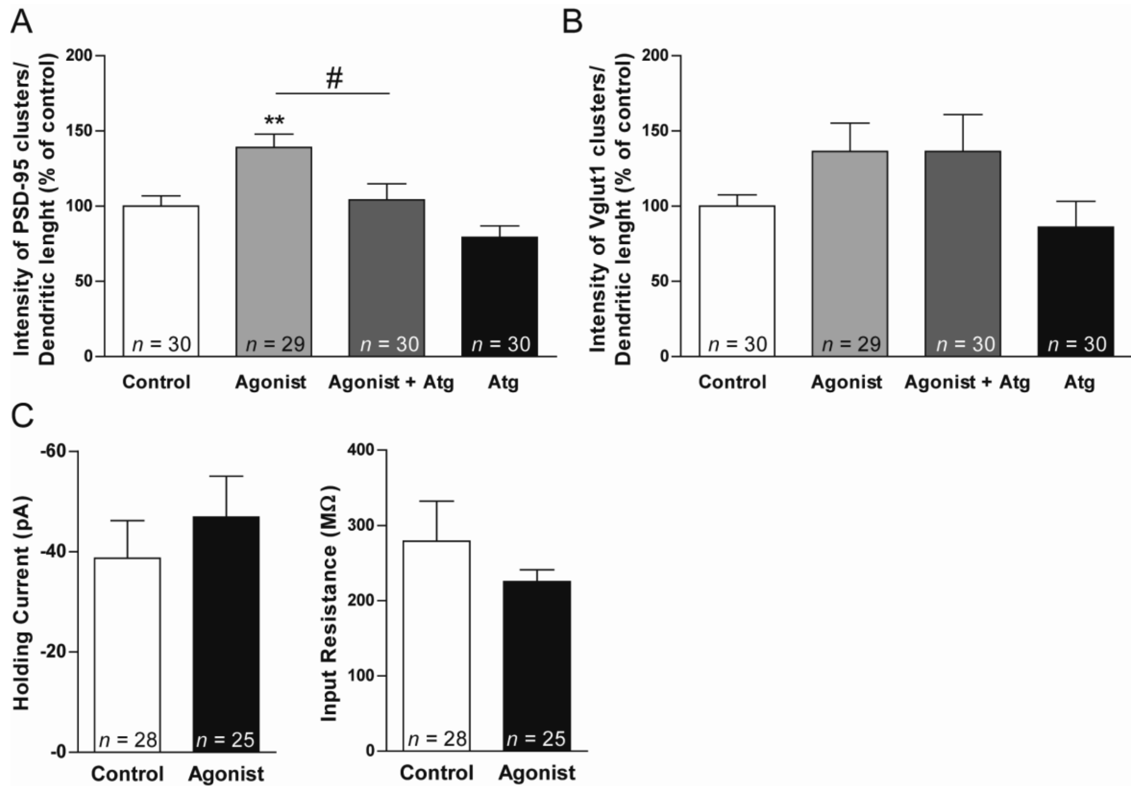

Fig. S2. Ghrelin receptor activation increases the total fluorescence intensity of PSD-95. (**A**,**B**) Hippocampal neurons 19 DIV were incubated with the ghrelin receptor agonist MK-0677 (1 μM) for 1 h, with the agonist in the presence of the ghrelin receptor antagonist [D-Lys^3]-GHRP-6 (Atg, 100 μM), or with the antagonist (Atg) alone. After fixation, neurons were stained for PSD-95, Vglut1 and MAP2. Scale bars represent 2 μm. Neurons were analyzed for the PSD-95 total fluorescence intensity (**A**) and for the Vglut1 total fluorescence intensity (**B**) per dendritic length. Results are expressed as % of control cells, and are averaged from three independent experiments. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test (**$P < 0.01$). Comparisons between pairs were performed using the Mann-Whitney test ($^{\#}P < 0.05$). *n* represents the total number of analyzed cells in independent preparations. (**C**) Effects of ghrelin receptor agonist MK-0677 treatment on passive membrane properties of CA1 hippocampal neurons. Application of MK-0677 to cultured hippocampal slices did not alter input resistance or holding current, when compared with untreated control neurons. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test. *n* represents the number of cells.

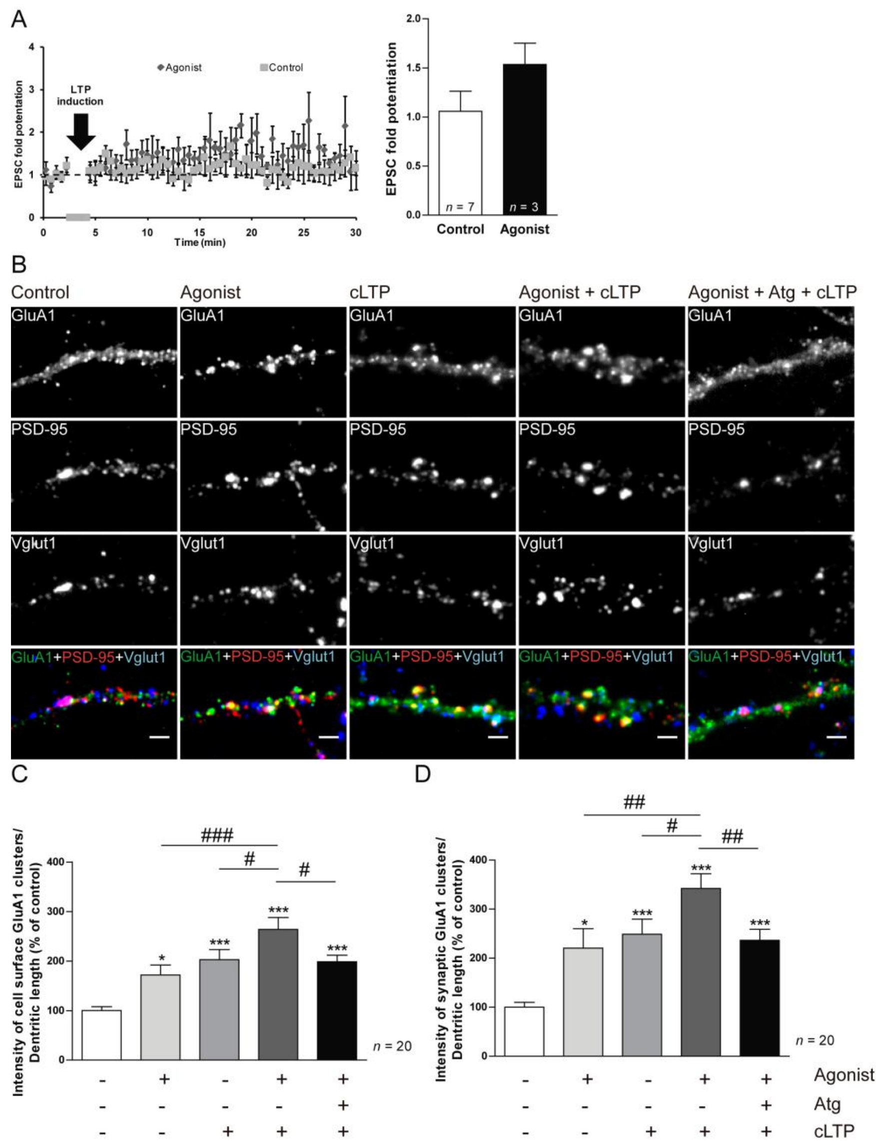

Fig. S3. Ghrelin receptor activation increases the chemical LTP-induced delivery of GluA1-AMPAR to synapses. (**A**) MK-0677 treatment did not have an effect on the non-potentiated (unpaired) pathway. Time course of normalized AMPAR-mediated synaptic responses for the unpaired pathway, before and after LTP induction. For simplicity, each time point in the plot corresponds to the average of 12 consecutive stimulations (sampling rate: 0.2 Hz). Organotypic slice cultures were incubated in culture medium, or in medium containing the ghrelin receptor agonist MK-0677 (1 μM, 20 h). Plot shows quantification of average synaptic potentiation from the unpaired pathway 20–30 min after LTP induction. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test. *n* represents the number of cells. (**B**) DIV 19 hippocampal neurons in culture were submitted to the following stimuli: ghrelin receptor agonist MK-0677 (1 μM, 20 h), chemical LTP (cLTP; 300 μM glycine for 3 min in the absence of Mg^{2+}), cLTP in neurons treated with the ghrelin receptor agonist MK-0677, or simultaneously with the ghrelin receptor agonist and antagonist [D-Lys^3]-GHRP-6 (Atg, 100 μM). Neurons were kept at 37ºC for 20 min (without glycine) and were live stained for GluA1 using an antibody against an extracellular epitope in the GluA1 N-terminus. After fixation, neurons were stained for PSD-95, Vglut1 and MAP2. Synaptic GluA1 was defined as the GluA1 signal overlapping with Vglut1. Scale bars represent 2 μm. Neurons were analyzed for the total GluA1 cell surface fluorescence intensity (**C**) and for the GluA1 synaptic cluster fluorescence intensity (**D**) per dendritic length. (**C**,**D**) Results are expressed as % of control cells, and are averaged from two independent experiments. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test (P < 0.0001) followed by the Dunn's Multiple Comparison test (*P<0.05 and P***<0.001). Comparisons between pairs were performed using the Mann-Whitney test ($^{\#}$P < 0.05, $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.001). *n* represents the number of cells.

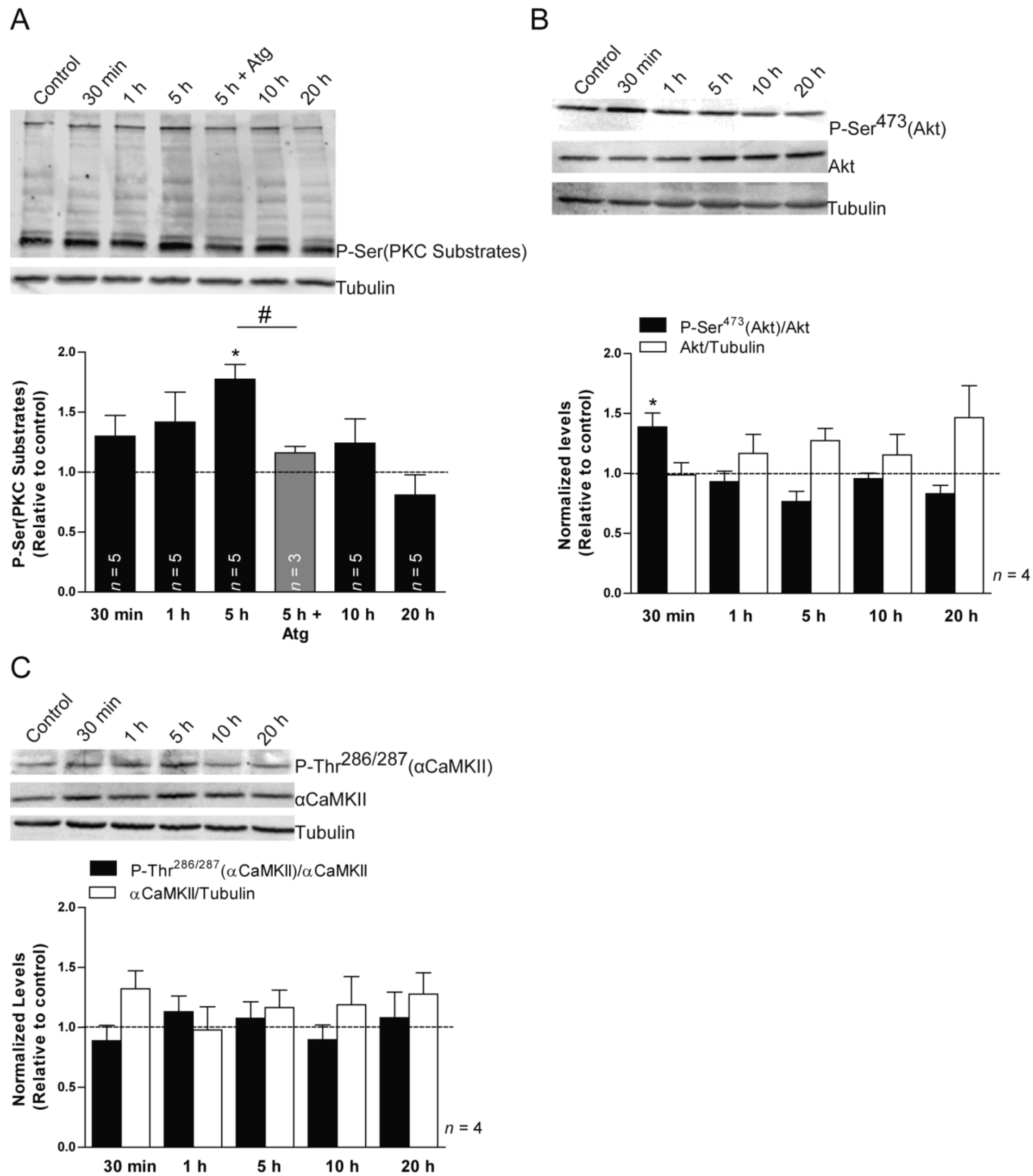

Fig. S4. Ghrelin receptor activation in organotypic hippocampal slices activates PKC and PI3 kinase signaling pathways. Western blot analysis of protein extracts from hippocampal slices incubated with culture medium or with medium containing the ghrelin receptor agonist MK-0677 (1 μM) for the indicated periods of time, or with MK-0677 in the presence of the ghrelin receptor antagonist [D-Lys^3]-GHRP-6 (Atg, 100 μM). The primary antibodies detected phosphorylation of PKC endogenous substrates with an antibody that recognizes a phospho-Ser PKC substrate motif (**A**), phosphorylation of Akt at Ser^{473} (**B**), a residue targeted by a PI3 kinase downstream signaling, and phosphorylation of αCaMKII at Thr$^{286/287}$ (**C**). Total Akt (**B**) and total αCaMKII (**C**) were also detected. Tubulin was used as a loading control in all cases. The graphs represent the quantification of band intensities relative to control extracts. Error bars represent s.e.m. The statistical significance was calculated using the Paired t test (*P < 0.05 and $^{\#}$P < 0.05). *n* represents the number of independent experiments.

Chapter 4

Constitutive activity of ghrelin receptor controls AMPA receptors-mediated transmission in the hippocampus

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In preparation for publication

Summary

Ghrelin is a peptide mainly produced by the stomach and released into circulation, affecting energy balance and memory retention. Herein, we explore the molecular mechanism underlying the hippocampal action of ghrelin on synaptic plasticity and learning, regarding the constitutive, ligand-independent activity of the ghrelin receptor. Our data show that blocking of ghrelin receptor constitutive activity decreases the AMPARs-mediated synaptic transmission in the hippocampus. Moreover, we show that ligand-independent activation of the ghrelin receptor triggers the phosphorylation of GluA1 and is developmentally regulated.

Introduction

Ghrelin is mainly a gastrointestinal hormone (Date et al., 2000a) and is the only circulating orexigenic factor secreted from a peripheral organ to act on the hypothalamus to regulate appetite and energy balance (reviewed in Kojima and Kangawa, 2010). The ghrelin receptor [or growth hormone secretagogue receptor 1a (GHS-R1a)] is a G protein-coupled receptor (GPCR) with a high degree of constitutive activity (presence of receptor signaling in the absence of any ligand) *in vitro* (Holst et al., 2003, 2006; reviewed in Mear et al., 2013). This ligand-independent binding activity is characteristic of GPCRs, and is particularly well characterized for serotonin and cannabinoid receptors (reviewed in Aloyo et al., 2010). Physiological relevance of the constitutive activity of GPCRs is steadily growing (reviewed in Bond and Ijzerman, 2006).

Constitutive signaling from the ghrelin receptor may also be important *in vivo* in the control of food intake and body weight, as recently demonstrated (Petersen et al., 2009). Moreover it has been implicated in a syndrome of short stature and obesity (reviewed in Holst and Schwartz, 2006). The function of ghrelin receptor constitutive activity in the hippocampus is largely unknown, however recently it was implicated in limbic seizure (Portelli et al., 2012).

The GHS-R1a receptor was initially found in the hypothalamus and in the pituitary gland (Howard et al., 1996), and later in the hippocampus (Guan et al., 1997). Consistent with this expression pattern, ghrelin and its receptor have been related with functions other than those related to endocrine and metabolic regulation (reviewed in Andrews, 2011). Indeed, in the last few years several studies have shown that ghrelin increases hippocampal-dependent memory retention in rodents, upon peripheral and central injection (Carlini et al., 2002, 2004; Diano et al., 2006; Chen et al., 2011). Moreover, ghrelin deficient mice exhibit decreased novel object recognition (Diano et al., 2006) and ghrelin receptor knockout mice display impairments in Morris water maze test (Davis et al., 2011), strongly suggesting that endogenous ghrelin receptor-mediated signaling has a physiological role in improving hippocampal-dependent learning and memory. However, despite this strong behavior-based evidence, the molecular mechanisms that underlie this function are still poorly understood.

Here, we have tested whether blockade of the ghrelin receptor constitutive activity affects the excitatory synaptic transmission in the hippocampus, which has been consistently suggested as the main cellular target for learning and memory phenomena, in particular between CA3 and CA1 pyramidal neurons (reviewed in Lüscher and Malenka, 2012). Using organotypic hippocampal slices, and recording synaptic responses from the CA3-CA1 synapse, we found that blockade of ligand-independent ghrelin receptor activation decreases the AMPA/NMDA ratio of synaptic responses. This strongly suggests that ghrelin receptor constitutive activity is involved in the maintenance of AMPARs-mediated synaptic transmission.

Moreover, this alteration was accompanied by a decrease in GluA1 phosphorylation in a residue involved in AMPARs-synaptic trafficking (serine residue at position 845 of GluA1 intracellular C-terminus). Finally, the ligand-independent ghrelin receptor activity in the hippocampus appears to be developmentally regulated.

Results

Blockade of the Constitutive Activity of GHS-R1a Decreases the AMPARs-Mediated Synaptic Transmission

High constitutive activity is an intrinsic feature of the ghrelin receptor (Holst et al., 2003, 2006; reviewed in Mear et al., 2013). To evaluate whether the constitutive activity of the ghrelin receptor affects glutamatergic transmission at the CA3-CA1 hippocampal synapse, we blocked it with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P, a potent inverse agonist for the constitutive ligand-independent signaling of GHS-R1a (Holst et al., 2003, 2006). 6 DIV organotypic hippocampal slices were treated with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P (1 μM) for 20 h, and electrophysiological recordings were performed at 7 DIV. The AMPA/NMDA ratio of synaptic responses significantly decreased after treatment with the inverse agonist compared with control neurons (Figure 1A), thus suggesting that the basal activity of the ghrelin receptor in the hippocampus controls AMPARs-mediated synaptic transmission. To determine whether ghrelin or other ligands for the ghrelin receptor [given that the minimal sequence required for binding and activation of GHS-R1a are the first 4 residues of ghrelin (conserved along mammals) (Bednarek et al., 2000)] in the culture medium could activate the receptor and thus affect glutamatergic transmission, hippocampal organotypic slices were chronically treated with the GHS-R1a antagonist [D-Lys^3]-GHRP-6 (100 μM) from 3 DIV up to 7 DIV, and electrophysiological recordings were performed at this stage. We found that AMPA/NMDA ratio did not change after antagonist treatment compared with control neurons (Figure 1A), suggesting that the influence of GHS-R1a on AMPARs-mediated synaptic transmission, which is blocked by the ghrelin receptor inverse agonist, is merely mediated by ligand-independent signaling of GHS-R1a.

To explore whether ghrelin receptor activation in response to the ligand might affect AMPARs-mediated synaptic transmission in 7 DIV hippocampal slices, in addition to the constitutive receptor activity-mediated component that we identified (Figure 1A), 6 DIV organotypic hippocampal slices were treated with the GHS-R1a agonist MK-0677 (1 μM) for 20 h, and electrophysiological recordings were performed at 7 DIV. The AMPA/NMDA ratio of synaptic responses did not change after MK-0677 treatment compared with control neurons (Figure 1B), thus suggesting that the ligand-dependent ghrelin receptor activation is incapable of further increasing AMPARs-mediated synaptic transmission in the hippocampus, at this stage of development, in contrast to what was observed for younger hippocampal neurons (Chapter 3).

We further evaluated whether treatment with the GHS-R1a agonist induces the delivery of new AMPARs into synapses in 7 DIV hippocampal slices, by expressing GFP-tagged GluA1 subunit in CA1 neurons. Overexpression of GluA1, with a Sindbis virus expression system,

leads to the formation of homomeric AMPARs containing the GluA1 subunit (Hayashi et al., 2000). These GluA2-lacking receptors are inwardly rectifying (Boulter et al., 1990; Hollmann et al., 1991; Verdoorn et al., 1991), and therefore their recruitment to the synapse can be monitored as an increase in the ratio of the evoked post synaptic current at –60 mV relative to the current at +40 mV [rectification index; (Hayashi et al., 2000)]. In this system it was previously shown that newly synthesized GluA1-containing AMPARs are not spontaneously inserted at synapses, unless driven by strong synaptic stimulation or activation of specific signaling pathways associated with LTP induction (Hayashi et al., 2000; Boehm et al., 2006). Organotypic slice cultures were infected with GluA1-GFP at 5 DIV, treated at 6 DIV, and electrophysiology recordings were performed at 7 DIV. Treatment of organotypic slices with the GHS-R1a agonist MK-0677 for 20 h did not change the rectification index in uninfected cells (Figure 1C), indicating that GHS-R1a activation in response to ligand does not alter the intrinsic rectification properties of endogenous AMPARs. In striking contrast to what we observed using younger hippocampal slices (Chapter 3), we found that slice treatment with MK-0677 did not change the rectification index in neurons that express GluA1-GFP (Figure 1C), suggesting that ligand-dependent ghrelin receptor activation does not induce synaptic delivery of AMPARs. Altogether these findings suggest that ligand-independent signaling through the GHS-R1a contributes to the basal maintenance of AMPARs-mediated synaptic transmission in the hippocampus at this stage of development.

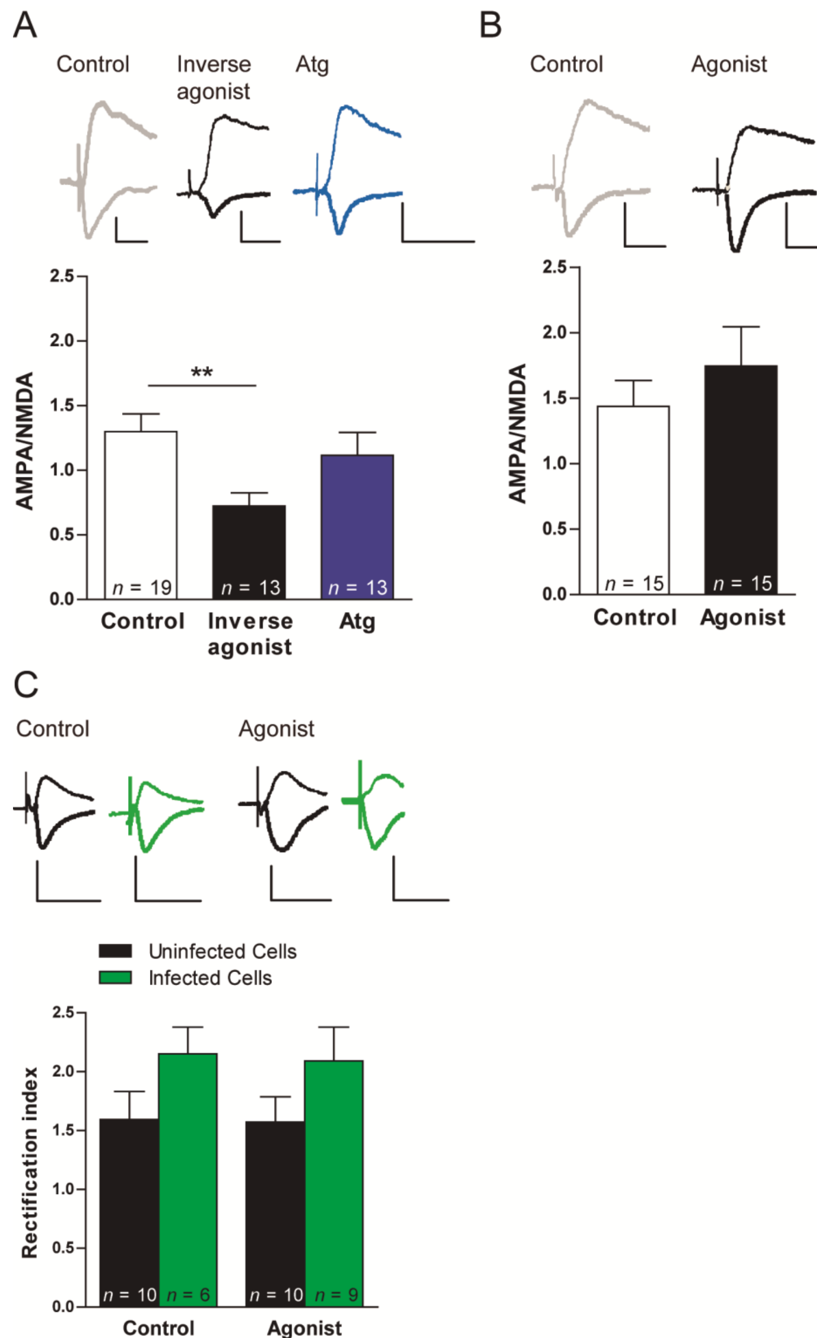

Figure 1. Treatment with an inverse agonist of ghrelin receptor decreases the AMPA/NMDA ratio in the hippocampus. (**A**,**B**) Comparison of evoked synaptic responses in organotypic hippocampal slices treated with the ghrelin receptor inverse agonist [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P (1 μM) for 20 h, with the ghrelin receptor antagonist [D-Lys[3]]-GHRP-6 (Atg, 100 μM) chronically, or treated with the ghrelin receptor agonist MK-0677 (1 μM) for 20 h. Average AMPA/NMDA ratios for inverse agonist-, antagonist-treated and control cells (**A**), and for agonist-treated and control cells (**B**). (**C**) Voltage-clamp whole cell recordings obtained in CA1 neurons expressing GluA1-GFP (Infected cells) and adjacent non-fluorescent neurons (Uninfected cells). GluA1 synaptic delivery was quantified as an increase in the rectification index (RI = I_{-60}/I_{+40}). Average RIs for untreated cells and ghrelin receptor agonist MK-0677-treated cells (1 μM, 20 h). (**A**–**C**) Representative traces appear above the corresponding bars. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test (**P < 0.01). *n* represents the number of cells. Scale bars: vertical, 50 pA; horizontal, 20 ms.

Blockade of the Constitutive Activity of GHS-R1a Decreases the Phosphorylation of GluA1

Interplay between phosphorylation and dephosphorylation is crucial for controlling AMPARs surface expression and consequently AMPARs-mediated synaptic transmission (reviewed in Henley et al., 2011). With this assumption in mind, we evaluated the effect of blockade of ghrelin receptor constitutive activity on two key events playing an important role in the trafficking of AMPARs: GluA1 and stargazin phosphorylation. These biochemical changes were evaluated in whole cell extracts from 7 DIV organotypic hippocampal slices after adding the GHS-R1a agonist MK-0677 (1 μM) and the GHS-R1a inverse agonist [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P (1 μM) for 20 h.

Phosphorylation of GluA1 at Ser^{831} and Ser^{845}, and stargazin at Ser$^{239/240}$ were evaluated with the corresponding phospho-specific antibodies. GluA1 phosphorylation at Ser^{831} [a protein kinase C (PKC) or calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) substrate] did not change after incubation with either agonist or inverse agonist (Figure 2A), while treatment with the inverse agonist significantly decreased phosphorylation in the Ser^{845} [a protein kinase A (PKA) or cGMP-dependent kinase II (cGKII) substrate] residue (Figure 2A). Additionally, we found that ghrelin receptor agonist treatment did not significantly change the phosphorylation levels of GluA1 in this residue (Figure 2A). These results suggest that the ligand-independent activity of ghrelin receptor triggers the activation of a signaling pathway whose substrate is Ser^{845} in the GluA1 C-terminus. Because it was found that in hypothalamic neurons (Kohno et al., 2003) and in organotypic hippocampal slices (Cuellar and Isokawa, 2011) the ghrelin receptor is coupled to a $G_s\alpha$ subunit of a G protein this signaling pathway might be through PKA. Thus, PKA appears to be responsible for the maintenance of a certain level of GluA1 phosphorylation at Ser^{845}; blockade of the constitutive activity of GHS-R1a with the inverse agonist therefore decreases GluA1 phosphorylation at Ser^{845}. However, at this developmental stage, the agonist-dependent ghrelin receptor activation is unable to further increase the phosphorylation levels of GluA1 in these residues. Moreover, treatment with either agonist or inverse agonist did not change the phosphorylation of stargazin at Ser$^{239/240}$ (a PKC or CaMKII substrate) (Figure 2B). Finally, we also analyzed the phosphorylation levels of Akt at Ser^{473}, a residue targeted by a PI3 kinase downstream signaling pathway. We did not observe changes in the phosphorylation of this residue upon incubation with either the agonist or the inverse agonist of the ghrelin receptor (Figure 2C). Altogether, these observations suggest that ligand-independent signaling of GHS-R1a triggers the PKA signaling pathway in the hippocampus, which results in tonic phosphorylation of GluA1 at Ser^{845}.

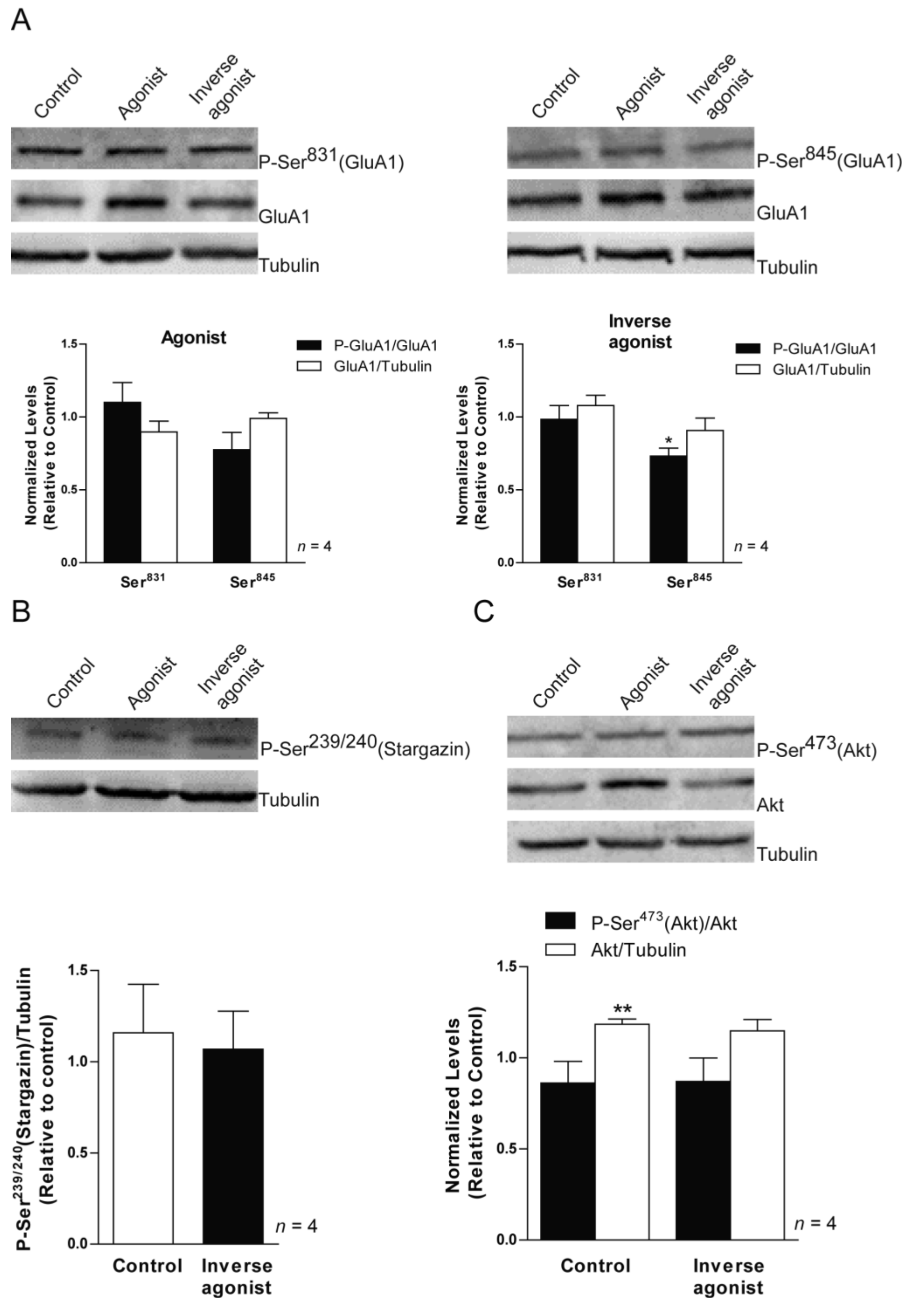

Figure 2. Treatment with an inverse agonist of ghrelin receptor decreases the phosphorylation of GluA1 in the hippocampus. Western blot analysis of protein extracts from hippocampal slices incubated with culture medium or medium containing the ghrelin receptor agonist MK-0677 (1 μM), or the ghrelin receptor inverse agonist [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P (1 μM) for 20 h. Primary antibodies detected phosphorylation of GluA1 at Ser^{831} and at Ser^{845} (**A**), phosphorylation of stargazin at Ser$^{239/240}$ (**B**) and phosphorylarion of Akt at Ser^{473} (**C**), a residue targeted by a PI3 kinase downstream signaling pathway. Total GluA1 (**A**) and total Akt (**C**) were also detected. Tubulin was used as a loading control in all cases. The graphs represent the quantification of band intensities relative to control extracts. Error bars represent s.e.m. The statistical significance was calculated using the Paired t test (*P < 0.05 and **P < 0.01). *n* represents the number of independent experiments.

Constitutive Activity of GHS-R1a is Developmentally Regulated

We also evaluated whether the ligand-independent signaling of GHS-R1a is developmentally regulated in the hippocampus. Organotypic hippocampal slices (2 or 3 DIV) were treated with the GHS-R1a inverse agonist [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P (1 μM) for 20 h, and whole cell extracts were prepared. We observed that, in opposition to what was seen for 7 DIV slices (Figure 2A), treatment with the inverse agonist did not affect the phosphorylation levels of GluA1 at Ser^{845} (Figure 3A). Moreover, at this developmental stage the agonist-dependent activation of GHS-R1a resulted in an increase on the phosphorylation of GluA1 at Ser^{845} (Figure 3A). These observations suggest that from 3 DIV up to 7 DIV there is an alteration in the ghrelin receptor-mediated signaling in the hippocampus. In younger stages of development, ghrelin receptor-mediated signaling affects AMPAR-mediated responses upon receptor activation by the ligand (Chapter 3), while later in development the constitutive activity of the ghrelin receptor seems to play a role in regulating AMPAR activity and GluA1 phosphorylation at the Ser^{845} PKA phosphorylation site.

Finally, we tested whether this alteration is correlated with changes in the expression of GHS-R1a during the development. GHS-R1a expression levels were evaluated in 3 or 4 DIV and 7 DIV organotypic hippocampal slices. We observed that GHS-R1a expression levels significantly decreases during this short time window (Figure 3A), thus suggesting that the increase in the ligand-independent signaling of GHS-R1a observed at 7 DIV compared to 3-4 DIV is not secondary to an increase on the ghrelin receptor expression levels.

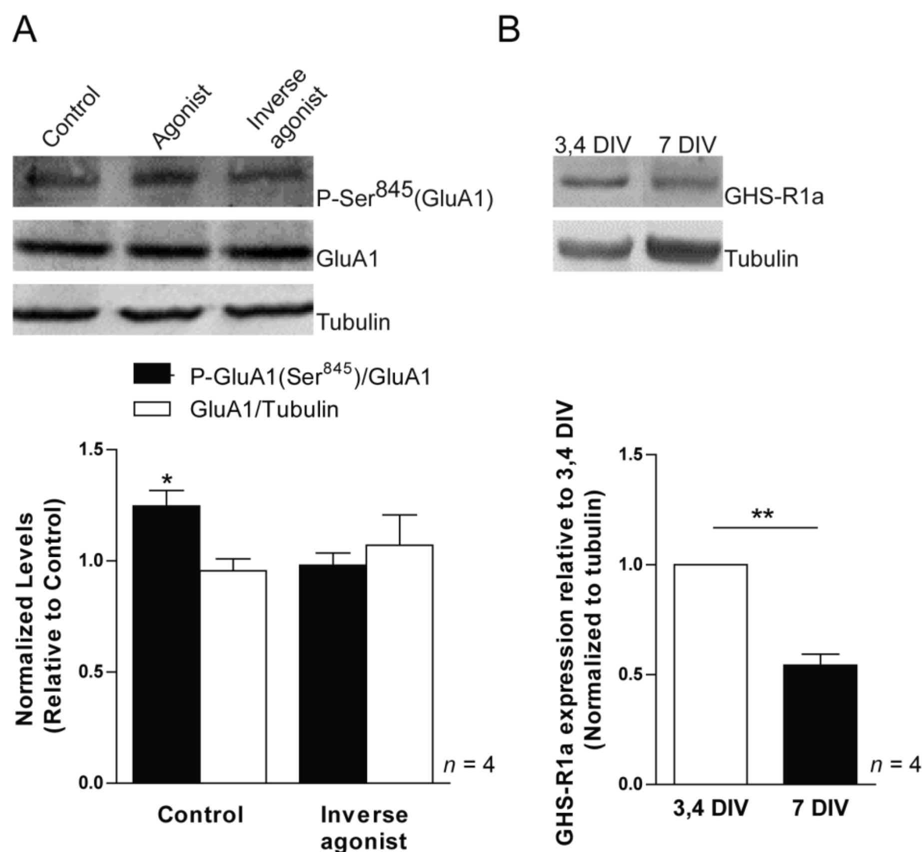

Figure 3. Treatment with an inverse agonist of the ghrelin receptor does not affect the phosphorylation in the Ser^{845} of GluA1 in younger hippocampal slices. (**A**) Western blot analysis of protein extracts from hippocampal slices incubated with culture medium or medium containing the ghrelin receptor agonist MK-0677 (1 μM), or the ghrelin receptor inverse agonist [D-Arg^{1}, D-Phe^{5}, D-$Trp^{7,9}$, Leu^{11}]-substance P (1 μM) for 20 h. The primary antibodies detected phosphorylation of GluA1 at Ser^{845} and total GluA1. The graph represents the quantification of band intensities relative to control extracts. Tubulin was used as a loading control (**B**) Developmental profile for the expression of GHS-R1a in organotypic hippocampal slices. GHS-R1a expression levels decrease from 3,4 DIV to 7 DIV. The plot represents the mean intensity of GHS-R1a bands normalized to tubulin, relative to 3,4 DIV. (**A**,**B**) Error bars represent s.e.m. The statistical significance was calculated using the Paired t test (*P < 0.05 and **P < 0.01). *n* represents the number of independent experiments.

Constitutive Activity of GHS-R1a is Involved in the Developmental Acquisition of AMPAR-Mediated Synaptic Transmission

Anatomical and electrophysiological experiments show that central excitatory synapses initially display NMDARs and subsequently mature by acquiring AMPARs (Durand et al., 1996; Wu et al., 1996; Isaac et al., 1997; Nusser et al., 1998; Petralia et al., 1999). It is well established in the hippocampus (Durand et al., 1996; Hsia et al., 1998; Zhu and Malinow, 2002) and in other brain regions (Wu et al., 1996; Isaac et al., 1997) that the ratio of synaptic AMPARs responses to synaptic NMDARs responses increases rapidly during the first postnatal week, with little change during the second week. In agreement, we found that in untreated organotypic hippocampal slices there is a significant increase in AMPA/NMDA ratio of synaptic responses after 3–4 days in culture (Figure 4). However, this developmental increase in AMPARs synaptic

responses is completely blocked by the ghrelin receptor inverse agonist (Figure 4). 7 DIV organotypic hippocampal slices after treatment with the ghrelin receptor inverse agonist [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P (1 μM) for 20 h have the same phenotype as 3 or 4 DIV slices. Thus, suggesting that the ligand-independent signaling of GHS-R1a, eventually triggering GluA1 phosphorylation, controls the maturation of excitatory circuits in the hippocampus, in particular the acquisition of developmentally-dependent AMPARs-mediated synaptic transmission.

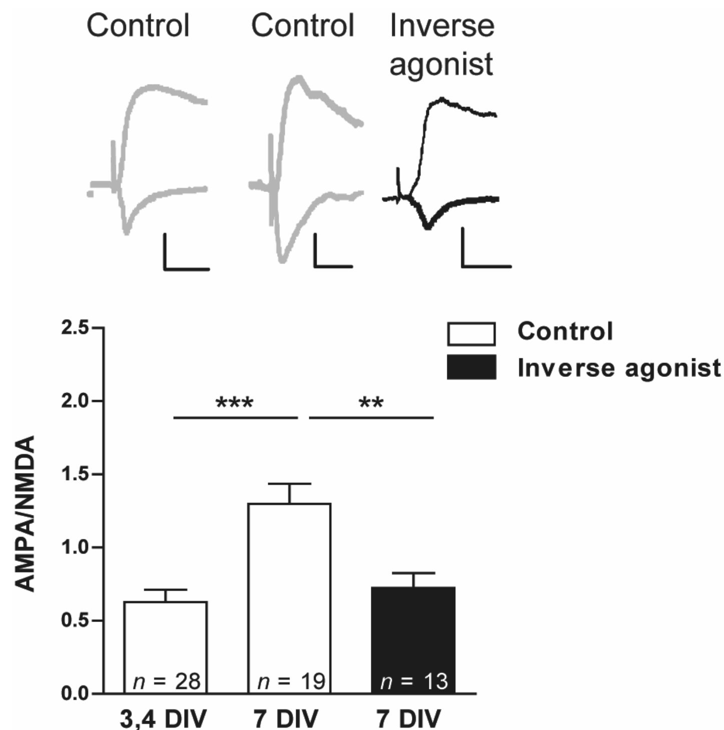

Figure 4. Treatment with an inverse agonist of the ghrelin receptor decreases the AMPA/NMDA ratio during development in the hippocampus. Comparison of evoked synaptic responses in untreated organotypic hippocampal slices with 3 or 4 DIV and 7 DIV and 7 DIV organotypic hippocampal slices treated with the ghrelin receptor inverse agonist [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P (1 μM) for 20 h. Average AMPA/NMDA ratios for untreated and inverse agonist-treated cells. Representative traces appear above the corresponding bars. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test (**P < 0.01 and ***P < 0.001). *n* represents the number of cells. Scale bars: vertical, 50 pA; horizontal, 20 ms.

Discussion

The role of ghrelin in hippocampal-dependent cognition is very well established. It was demonstrated that when ghrelin is peripherally or centrally administered there is an improvement in the performance of rodents in hippocampal-dependent behavior tests (Carlini et al., 2002, 2004; Diano et al., 2006; Chen et al., 2011). On the other hand, ghrelin knockout mice showed impaired performance in novel object recognition test (Diano et al., 2006) and ghrelin receptor knockout mice displayed impairments in the Morris water maze test (Davis et al., 2011), thus strongly suggesting a role for ghrelin receptor-mediated signaling in modulating hippocampal-dependent memory.

Although the function of ghrelin as a cognitive enhancer is well documented, the molecular mechanisms that underlie this function are still poorly understood. To address this question and given the particularly high constitutive activity of the ghrelin receptor (Holst et al., 2003, 2006), we evaluated the effect of ghrelin receptor constitutive activity blockade on the AMPARs-mediated synaptic transmission, as well as on the phosphorylation of GluA1. We found that upon blocking ligand-independent activation of ghrelin receptor there is a decrease in the AMPA/NMDA ratio of synaptic responses recorded at the CA3-CA1 synapse in organotypic hippocampal slices, thus suggesting a decrease in AMPARs-mediated synaptic transmission. Moreover, these changes were correlated with a decrease in the phosphorylation of GluA1, in the Ser^{845} residue. These data suggest that ligand-independent activity of the ghrelin receptor triggers activation of signaling pathway(s), which are essential to maintain GluA1 phosphorylation (which occurs in control conditions), necessary for AMPARs synaptic trafficking. Hence, in the presence of the ghrelin receptor inverse agonist, the phosphorylation of GluA1 mediated by activation of ghrelin receptor agonist-independent signaling is decreased, resulting in depression of AMPARs-mediated synaptic transmission.

Furthermore, we found that the ligand-independent contribution of the ghrelin receptor mediated-signaling to GluA1 phosphorylation is developmentally regulated. At younger stages of development, ligand-dependent ghrelin receptor activation increases the phosphorylation of GluA1, whereas the ligand-independent activity does not contribute for the overall GluA1 phosphorylation. On the contrary, at older stages of development, the ghrelin receptor activation, by ligand application, does not affect the phosphorylation of GluA1, which is decreased upon blockade of the ghrelin receptor constitutive activity. These results suggest that the agonist-independent component of ghrelin receptor-mediated signaling increases during development, at least when monitored through its effects on the phosphorylation levels of a PKA substrate (Ser^{845}). Of particular interest, we report for the first time that the constitutive activity of GHS-R1a is required for the maintenance of AMPARs-mediated synaptic transmission in the hippocampus.

The number of GPCRs displaying constitutive activity with clear physiological implications is not vast, but it is steadily growing (reviewed in Bond and Ijzerman, 2006). In the particular case of the ghrelin receptor, it has been proposed that selective loss of ghrelin receptor constitutive activity causes a syndrome of short stature and obesity (reviewed in Holst and Schwartz, 2006). Recently, it was also found that the blockade of ghrelin receptor constitutive activity in the hippocampus leads to attenuation of limbic seizures *in vivo* and epileptiform activity *in vitro*, suggesting that the inverse agonist for the ghrelin receptor is capable of exerting anticonvulsant effects (Portelli et al., 2012), and showing that the ghrelin receptor constitutive activity is relevant in the hippocampus.

Holst and co-workers found in an heterologous system that transfection of GHS-R1a resulted in a dose-dependent stimulation of the cAMP-responsive element (CRE) (Holst et al., 2003, 2004). Moreover, it was found that intracerebroventricular injection of [D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11}]-substance P in rodents inhibited food intake, preventing the body weight gain observed in control groups (Petersen et al., 2009). Additionally, these authors found that the blockade of the ghrelin receptor constitutive activity decreases the phosphorylation of CRE-binding protein (CREB) in a hypothalamic cell line (Petersen et al., 2009).

Since CREB is a major substrate for PKA (reviewed Sands and Palmer, 2008), and the evidence suggesting that ghrelin receptor is coupled to a $G_s\alpha$ subunit of a G protein in hypothalamus and hippocampus (Kohno et al., 2003; Cuellar and Isokawa, 2011), these results suggest that blocking ghrelin receptor constitute activity inhibits the PKA signaling pathway in the hippocampus. However, it is impossible to rule out the involvement of downstream kinases of the $G_{q/11}\alpha$-mediated signaling pathway, such as Ca^{2+}/calmodulin kinase IV (CaMK IV) or protein kinase C (PKC), which were also implicated in CREB phosphorylation (Matthews et al., 1994; Singh et al., 2001). These observations suggest that it may be worth to investigate whether CaMK IV is capable to phosphorylate the GluA1 C-terminus in the Ser^{845}. However, these results also suggest that the effect of blocking ghrelin receptor constitutive activity with the inverse agonist [D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11}]-substance P in the hippocampus, in the presence of a selective inhibitor of $G_{q/11}\alpha$ type of G proteins (Takasaki et al., 2004), should be investigated. These experiments would demonstrate categorically whether it is the PKA signaling which is being triggered by ghrelin receptor agonist-independent signaling.

Activation of PKA is very well established as a relevant factor for synaptic insertion of AMPARs. Esteban and co-workers found that PKA activity is necessary for the synaptic incorporation of GluA1 triggered by CaMKII activation (Esteban et al., 2003), thus suggesting that phosphorylation of GluA1 by PKA is necessary but not sufficient for its synaptic incorporation. It has been suggested that AMPARs are inserted into the plasma membrane in the soma or dendrites at extrasynaptic sites (Passafaro et al., 2001; Adesnik et al., 2005; Yudowski et al., 2007; Lin et al., 2009), followed by their incorporation into synapses. Oh and

co-workers further demonstrated the importance of PKA activity and phosphorylation of Ser^{845} in synaptic trafficking of AMPARs. They found that in response to forskolin/rolipram application (which elevates the endogenous cAMP by stimulating its production with the adenylate cyclase activator, forskolin, and preventing its degradation by the phosphodiesterase inhibitor, rolipram) AMPARs specifically traffic to extrasynaptic sites (Oh et al., 2006). This extrasynaptic trafficking occurs in the presence of the NMDARs antagonist AP5 and strongly correlates with the phosphorylation state of Ser^{845} in the GluA1 subunit, indicating that the trafficking to extrasynaptic sites is not NMDAR-dependent (Oh et al., 2006). Moreover, co-activation of cAMP signaling and synaptic NMDARs enhances the incorporation of AMPARs into synapses (Oh et al., 2006). Thus, Ser^{845} phosphorylation primes AMPARs for synaptic potentiation by trafficking them to extrasynaptic sites, possibly followed by synaptic incorporation requiring synaptic activity (e.g., NMDARs activity) (Oh et al., 2006).

Here we showed that when the ligand-independent ghrelin receptor activity is blocked in 7 DIV hippocampal slices there is a decrease in the AMPA/NMDA ratio of synaptic responses, suggesting that in the absence of the constitutive GHS-R1a signaling there is a depression in the AMPARs-mediated synaptic currents. This alteration is paralleled by a decrease in the phosphorylation of Ser^{845}. Thus we hypothesize that ligand-independent activation of ghrelin receptor-mediated signaling (through PKA activation) contributes for the maintenance of the critical pool of extrasynaptic GluA1-containing AMPARs, which upon synaptic activity are synaptically inserted. Hence, in the presence of the ghrelin receptor inverse agonist, comparing with the control conditions (where the PKA is being activated by GHS-R1a constitutive activity), there is a decrease in GluA1 phosphorylation, which consequently disrupts GluA1 trafficking to extrasynaptic sites and causes depression of AMPARs-mediated synaptic currents.

The cAMP-dependent activation of PKA upon ligand-dependent ghrelin receptor activation had already been demonstrated in the hippocampus (Cuellar and Isokawa, 2011), suggesting that in the hippocampus this type of GPCR is coupled to the $G_s\alpha$ type of G proteins. Here we also suggest, for the first time in this region, that the constitutive activity of the ghrelin receptor might be also coupled to the activation of PKA, probably via cAMP production, consistent with what happens in an heterologous system (Holst et al., 2003, 2004) and in an hypothalamic cell line (Petersen et al., 2009). However, this observation needs further experimental validation.

Here we also showed that this regulation of PKA activity via agonist-independent ghrelin receptor activation is developmentally regulated in the very short, but critical, time window from 3 DIV up to 7 DIV. This suggests an involvement of the constitutive activity of ghrelin receptor in the development of excitatory circuits in the hippocampus. It has been suggested that the AMPA/NMDA ratio of synaptic responses increases rapidly during the first postnatal week, with little change during the second week (Durand et al., 1996; Hsia et al., 1998; Zhu and Malinow,

2002). NMDARs activity is essential for the rapid delivery of AMPARs in glutamatergic synapses during development (Zhu and Malinow, 2002). Hence, ligand-independent activation of the ghrelin receptor may increase the phosphorylation of GluA1 at Ser^{845}, increasing the trafficking of GluA1-containing AMPARs to extrasynaptic sites. Further activation of NMDARs will promote synaptic insertion, with consequent increase in the AMPA/NMDA ratio. These observations suggest a role for the constitutive activity of the ghrelin receptor in increasing the AMPARs-mediated synaptic currents during this critical period of development.

We also showed that this apparent increase in the constitutive activity of the ghrelin receptor is not correlated with an increase in the expression of the ghrelin receptor. Indeed, we observed a decrease in the expression of the ghrelin receptor within the period analyzed. It has been proposed that the heterodimerization of the ghrelin receptor with its truncated form (GHS-R1b) attenuates its constitutive activation due to a decrease in cell surface expression (Leung et al., 2007; Chow et al., 2012). In addition, heterodimerization with other GPCRs was also observed. For instance, it was found that the vast majority of GHS-R1a expressing neurons in the arcuate nucleus (ARC) also express melanocortin-3 receptor (MC3R) (Rediger et al., 2009). Rediger and co-workers observed that the basally active conformation of GHS-R1a increases the capacity of MC3R for $G_s\alpha$ activation and cAMP accumulation after melanocyte stimulating hormone (MSH) binding. In an heterologous system the interaction of GHS-R1a with MC3R results in hyper-stimulation of the MSH-induced cAMP signaling pathway, an effect which is blocked by increasing amounts of [D-Arg^{1}, D-Phe^{5}, D-$Trp^{7,9}$, Leu^{11}]-substance P (Rediger et al., 2009). These observations suggest that the constitutive activity of the ghrelin receptor might modulate the functional properties of a second GPCR in a dimmer with it. Heterodimerization of ghrelin receptor with other GPCRs in the hippocampus is completely unknown. Our study suggests that it may be worth to investigate whether the presence of ghrelin receptor heterodimers in the hippocampus, and whether these heterodimers affect the cAMP-dependent signaling pathways, in particular during development.

In conclusion, this study gives new molecular insights into how the ghrelin receptor modulates hippocampal-dependent glutamatergic transmission. In particular, our data suggest that the ligand-independent activity of the ghrelin receptor, through phosphorylation of GluA1, has a role in maintaining a population of phosphorylated GluA1-containing AMPARs.

Chapter 5

General Discussion and Future Directions

Changes in AMPARs and Stargazin Phosphorylation upon Ghrelin Receptor Activation – a Mechanism for the Potentiation of AMPARs-Mediated Synaptic Transmission

Here, we report that the activation of GHS-R1a with the agonist MK-0677 significantly increases the phosphorylation of serine residues 831 (Ser^{831}) and 845 (Ser^{845}) in the C-terminal region of GluA1. Moreover, we also found that ghrelin receptor activation increases the phosphorylation of the AMPARs-associated protein stargazin, and the activation of PKC and PI3 kinase signaling pathways.

These alterations were paralleled by changes in the trafficking of endogenous GluA1-containing AMPARs and homomeric GluA1-GFP AMPARs, causing enhancement of excitatory synaptic transmission (when assessed by the ratio of AMPA/NMDA of synaptic responses). Because the NMDA/GABA ratio of synaptic responses was not changed, we believe that GHS-R1a activation produces a functional change at excitatory CA1 synapses, specifically an increase in AMPARs-mediated synaptic transmission.

Moreover, we also found that GHS-R1a activation, upon application of MK-0677, dramatically enhanced NMDARs-dependent LTP expression in the CA3-CA1 synapse of the hippocampus, and increased the NMDARs-triggered delivery of endogenous GluA1-containing AMPARs to synaptic sites, when assessed in a neuronal culture model of LTP. These findings suggest that GHS-R1a activation in the hippocampus increases the GluA1-containing AMPARs delivery to synapses, thus facilitating the expression of LTP-like events.

According to the literature, two major events are required for GluA1-containing AMPARs synaptic insertion: extrasynaptic insertion followed by synaptic clustering upon NMDARs activation. Similarly, taking our results in consideration we herein propose that GHS-R1a activation increases the extrasynaptic delivery of AMPARs, which by the ongoing NMDARs-mediated synaptic activity are later synaptically inserted, enhancing AMPARs-mediated synaptic transmission in the hippocampus. Two major findings support this hypothesis: i) GHS-R1a activation increases the phosphorylation of GluA1 and stargazin and ii) the increase in GluA1-GFP synaptic insertion is blocked by inhibition of both NMDARs and cell spontaneous activity.

Hence, GHS-R1a activation in the CA1 region of the hippocampus increases the phosphorylation of Ser^{845} in the GluA1 C-terminus, eventually through activation of PKA. It was previously found that in the hippocampus the GHS-R1a is coupled to activation of this signaling pathway, through the production of cAMP (Cuellar and Isokawa, 2011). Additionally, it might also increase the phosphorylation of Ser^{816} and Ser^{818} in the GluA1 C-terminus (although, we have not directly assessed the phosphorylation in these two residues, we observed an increase

in the PKC signaling pathway, which is responsible for their phosphorylation). These biochemical alterations, as already reported (Ehlers, 2000; Esteban et al., 2003; Lee et al., 2003; Boehm et al., 2006; Oh et al., 2006; Lin et al., 2009), are necessary for GluA1-containing AMPARs trafficking, in particular for trafficking to extrasynaptic sites (Oh et al., 2006; Lin et al., 2009). However, these changes are not sufficient for the synaptic clustering of GluA1-containing AMPARs, which only occurs upon NMDARs-activation (Esteban et al., 2003; Oh et al., 2006).

It has been proposed that NMDARs-mediated activity is necessary for the synaptic recruitment of CaMKII. Once in the PSD, CaMKII phosphorylates the AMPARs-auxiliary protein stargazin, facilitating its binding to PSD-95, thereby indirectly stabilizing GluA1-contanining AMPARs (Opazo et al., 2010). In line with this idea, once in extrasynaptic sites, these receptors might be synaptically immobilized by CaMKII-mediated activity. On the other hand, it was found that under spontaneous activity CaMKII is also present at the synapse, in a NMDARs-dependent manner (Bayer et al., 2006). Hence, as mentioned above, the GHS-R1a activation promotes the extrasynaptic insertion of GluA1-containing AMPARs, which by the ongoing NMDARs-mediated synaptic activity are later synaptically inserted, satisfying the requirement for spontaneous activity and NMDARs-mediated activity.

Additionally, GHS-R1a activation might also directly modulate this final step of synaptic insertion (or stabilization at the synapse) of GluA1-containing AMPARs. We observed that upon agonist application there is a significant increase in the phosphorylation of the AMPARs-auxiliary protein stargazin, in two residues that are substrate for PKC, in addition to CaMKII (Tomita et al., 2005b). Additionally, we also observed an increase in the activation of PKC signaling pathway, thus suggesting that PKC is the signaling pathway responsible for stargazin phosphorylation upon GHS-R1a activation in the hippocampus here observed. These observations suggest that through phosphorylation of stargazin GHS-R1a activation may also increase synaptic clustering of GluA1-contanining AMPARs which are anchored by stargazin and PSD-95.

One last observation suggests an additional mechanism for the role of GHS-R1a in the synaptic immobilization of AMPARs. We also found that GHS-R1a activation, upon treatment with the agonist MK-0677, significantly increased the phosphorylation of Akt in the serine residue 473 (Ser^{473}), a residue targeted by a PI3 kinase downstream effector, suggesting an increase in the PI3 kinase signaling pathway. This is in agreement with Chen and co-authors who found that infusion of ghrelin into the dentate gyrus increased phosphorylation in this residue, in a time dependent-manner (Chen et al., 2011). It is well established that PI3 kinase cascade regulates the induction (Opazo et al., 2003) and/or expression of LTP (Sanna et al., 2002), being required for AMPARs synaptic insertion (Man et al., 2003). It was proposed that PI3 kinase, by means of its association with AMPARs (Man et al., 2003), is responsible for a constant supply of PIP3 necessary for the acumulation of PSD-95 at spines, therefore

preventing GluA1-containing AMPARs dispersion into the neighboring extrasynaptic membranes of the spine (Arendt et al., 2010). In agreement with this idea, upon GHS-R1a activation we also observed an increase in the total intensity of PSD-95 clusters. An increase in the levels of PSD-95 might be necessary to sustain the increase of GluA1-containing AMPARs at the synaptic sites.

Interestingly, these biochemical changes and consequent basal AMPARs-mediated synaptic transmission potentiation do not saturate (or occlude) further LTP expression. In fact, LTP expression in organotypic hippocampal slices and delivery of GluA1-containing AMPARs induced by chemical LTP in hippocampal cultured neurons are both enhanced upon GHS-R1a activation. Indeed, we found that GHS-R1a activation and chemical LTP induction may activate different signaling cascades (or its activation does not saturate or occlude the signaling pathways triggered by NMDARs activation), because the fluorescence intensity of synaptic GluA1 surface clusters in hippocampal neurons treated with the GHS-R1a agonist MK-0677, and pre-treated with the agonist and subjected to chemical LTP induction were significantly different.

These considerations lead us to propose a model (Figure 1) in which ghrelin receptor activation triggers signaling pathways which act on targets that facilitate LTP expression. It has been suggested that GluA1 phosphorylation plays a critical role in the expression of LTP, but not in its induction, because LTP is not completely absent in the double phosphomutants for GluA1 Ser^{845} and Ser^{831} phosphorylation sites (Lee et al., 2003). Synaptically inserted AMPARs require phosphorylation of GluA1 subunit to stay at the synapse (Lee et al., 2003). In the presence of the ongoing neuronal activity, in particular NMDARs-mediated activity, ghrelin receptor activation, which increases AMPARs and stargazin phosphorylation, enhances synaptic clustering of AMPARs (and its stabilization at the synapses), therefore causing potentiation of AMPARs-mediated synaptic responses. This strengthening of excitatory connections may in turn facilitate the expression of LTP-like events.

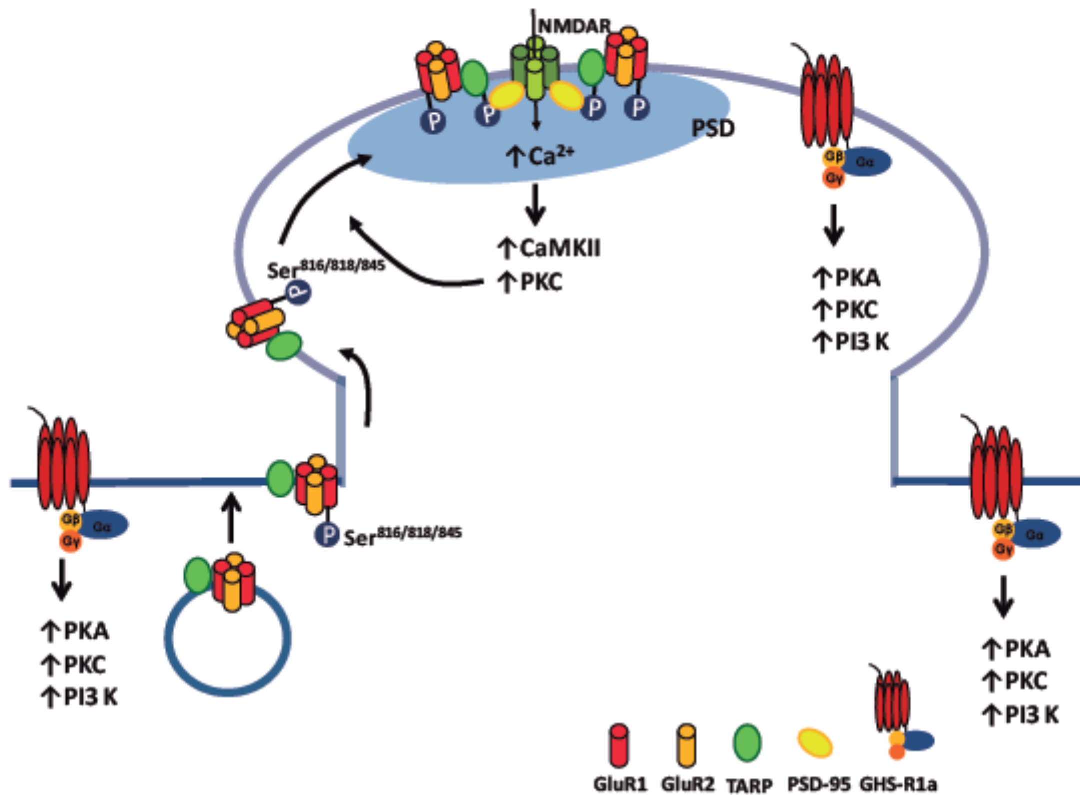

Figure 1. Model of excitatory synaptic transmission enhancement mediated by ghrelin receptor (GHS-R1a) activation. The GHS-R1a is localized postsynaptically in the vicinity of glutamatergic synapse, therefore in a strategic position to modulate the glutamatergic synaptic transmission. Ghrelin receptor activation triggers PKA and PKC signaling pathways which phosphorylate GluA1 C-terminus in the residues Ser^{845}, Ser^{816} and Ser^{818}. These biochemical alterations cause GluA1-containing AMPARs trafficking to extrasynaptic sites. At basal state, GluA1-containing AMPARs are highly mobile at neuronal surface. However, upon NMDARs activation, Ca^{2+}/CaM activates CaMKII inducing its synaptic insertion. CaMKII phosphorylates the AMPARs auxiliary protein stargazin (TARP) facilitating its binding to PSD-95 and synaptic retention, thereby indirectly stabilizing GluA1-containing AMPARs at synapses. Two other events facilitate GluA1-containing AMPARs synaptic insertion. GHS-R1a activation might directly mediate: i) stargazin phosphorylation (through PKC-mediated activity) and ii) PSD-95 synaptic clustering, necessary to sustain the increase in AMPARs synaptic insertion (through PI3 kinase-mediated activity). Altogether these biochemical alterations facilitate the GluA1-containing AMPARs synaptic insertion upon phenomena of synaptic plasticity, such as long-term potentiation and chemical long-term potentiation. [Adapted from (Santos et al., 2009)].

The Effect of GHS-R1a Activation on AMPARs-mediated Synaptic Transmission is Developmentally Regulated

We found that the effect of GHS-R1a activation with the agonist is lost in older organotypic hippocampal slices, i.e., at 3 or 4 DIV agonist-dependent activation of GHS-R1a increases the AMPARs-mediated synaptic transmission, whilst at 7 DIV agonist-dependent activation of GHS-R1a is incapable of affecting AMPARs-mediated synaptic transmission (and the trafficking of GluA1-GFP homomeric AMPARs to synaptic sites). Moreover, we also observed that at 7 DIV blockade of the constitutive activity of GHS-R1a depresses

glutamatergic synaptic transmission (when assessed by the ratio of AMPA/NMDA of synaptic responses). At this stage we cannot rule out the possibility of an increase in the NMDARs-mediated currents, but these results suggest that most probably upon blockade of ligand-independent GHS-R1a-mediated signaling there is a decrease in the AMPARs-mediated synaptic transmission in older slices. Our assumption is further validated by the fact that depression in glutamatergic synaptic transmission was paralleled by a decrease in phosphorylation of Ser^{845} in GluA1 C-terminus.

Our findings suggest that from 3 or 4 DIV up to 7 DIV there is a dramatic change in the effect of GHS-R1a-mediated signaling on AMPARs-mediated synaptic transmission. In other words, at 3 or 4 days in culture the effect of GHS-R1a activation on AMPARs-mediated synaptic transmission is dependent on GHS-R1a activation by the ligand, whilst at 7 days in culture the effect of GHS-R1a activation on AMPARs-mediated synaptic transmission appears to be mediated by the constitutive activity of the receptor. To the best of our knowledge, this change in the activation pattern of a GPCR during development, with consequences on glutamatergic synaptic transmission, has never been reported. Nevertheless, one important experiment that we have not yet performed is to test the effect of GHS-R1a constitutive activity blockade on AMPARs-mediated synaptic transmission in younger slices.

Another observation further supports this model. We found that blockade of GHS-R1a constitutive signaling at older slices decreases the phosphorylation of Ser^{845} in the GluA1 C-terminus (suggesting a decrease in the PKA signaling pathway), without affecting any of the other signaling pathway which we observed to be increased in younger slices upon treatment with the GHS-R1a agonist. Additionally, we found that agonist application to older slices is incapable of further increasing these signaling pathways (PI3 kinase, PKC – through its substrates GluA1 and stargazin). These results suggest that the constitutive and ligand-dependent activity of GHS-R1a are regulated during development in the hippocampus, and accompanied by activation of different signaling pathways. These alterations might be related with structural modifications of the receptor, because the constitutive activity of GHS-R1a appears to be related with structural aspects of the receptor (Holst et al., 2004). In addition, it may depend on its heterodimerization with its truncated splicing form (GHS-R1b), which seems to limit its surface expression, attenuating its constitutive activation (Leung et al., 2007; Chow et al., 2012); or with other GPCRs differentially expressed in the hippocampus throughout development and which might change its signaling properties (e.g., Rediger et al., 2009). These observations suggest that it may be worth investigating the presence of ghrelin receptor heterodimers in the hippocampus, and whether these heterodimers affect the signaling pathways of GHS-R1a in the dimer.

The enhancement of AMPARs-mediated synaptic transmission induced by ligand-dependent activation of GHS-R1a (discussed in detail above), which occurs in younger slices,

and the requirement of GHS-R1a constitutive activity for the maintenance of glutamatergic synaptic transmission, which occurs in older slices, not only highlight the importance of ghrelin-receptor mediated signaling in glutamatergic synaptic transmission in the hippocampus, but also suggest the involvement of the GHS-R1a-mediated signaling in the development of excitatory circuits in the hippocampus. It has been suggested that the AMPA/NMDA ratio of synaptic responses increases rapidly during the first postnatal week, with little change during the second week (Durand et al., 1996; Hsia et al., 1998; Zhu and Malinow, 2002). NMDARs activity is essential for the rapid delivery of AMPARs in glutamatergic synapses during development (Zhu and Malinow, 2002). Thus, initially, agonist-dependent GHS-R1a activation could increase the trafficking of GluA1-containing AMPARs to extrasynaptic sites, which are later synaptically inserted by NMDARs activation. Later in development, this mechanism of GHS-R1a might lose significance, and in turn the ligand-independent GHS-R1a activity is increased. Despite being a very short time window (3-7 DIV), this period is essential for the development of excitatory circuits in the hippocampus. In agreement with this role of GHS-R1a-mediated signaling in the modulation of glutamatergic synaptic transmission, we observed that GHS-R1a is developmentally regulated either in hippocampal cultures or in hippocampal slices. Consistent with this, a crucial experimental confirmation to demonstrate the involvement of both agonist-dependent and agonist-independent GHS-R1a activation in the modulation of hippocampal glutamatergic synaptic transmission in adult animals is still missing.

Finally, as indicated above, we observed a decrease in the phosphorylation levels of GluA1 at Ser^{845} upon blockade of the GHS-R1a constitutive activity. This down-regulation of GluA1 phosphorylation leads to depression of glutamatergic synaptic transmission (when assessed by the ratio of AMPA/NMDA of synaptic responses). We did not evaluate whether this depression is due to a decrease in the trafficking to extrasynaptic sites or due to an increase of GluA1 endocytosis, similarly to what happens during LTD, which is associated with dephosphorylation at Ser^{845} (Lee et al., 2000).

Life Without Ghrelin

A ghrelin knockout (KO) mice was produced, and its phenotype was examined (Sun et al., 2003; Wortley et al., 2004). Ghrelin knockout mice showed normal size, growth rate, food intake, body composition, reproduction, and gross behavior, without any pathological changes. Because survival is more acutely threatened by starvation than obesity, it is not surprising that an orexigenic-peptide-null mouse showed no change in food intake and body weight (reviewed in Kojima and Kangawa, 2005). However, the ghrelin-null mouse did not exhibit increased exploration time of the novel object, suggesting an impairment in their memory of the previous objects (Diano et al., 2006). Thus suggesting that endogenous ghrelin has a physiological role in improving learning and memory performances.

Mice lacking GHS-R1a (GHS-R1a-KO mice) do not show the typical increases in GH release and food intake upon ghrelin administration, indicating that GHS-R1a is indeed the primary biologically relevant ghrelin receptor (Sun et al., 2004). Growth and development of GHS-R1a-KO mice are normal, and their appetite and body composition do not differ from those of their wild-type littermates (Sun et al., 2004). However, in agreement with the ghrelin KO mice, Davis and co-workers found that GHS-R1a-KO mice displayed impairments in Morris water maze test.

This suggests that GHS-R1a-mediated signaling is not a critically required orexigenic factor, and may be more important for the regulation of higher brain functions, namely hippocampal-dependent learning and memory performance, as indicated by the behavioral tests. Patients after gastric bypass lose their weight, and their circulating ghrelin levels decrease to levels close to the detection limit (Cummings et al., 2002b). Moreover, plasma ghrelin levels in subjects who underwent gastric bypass did not oscillate between meals and were markedly lower than those of both lean controls and matched obese controls, altogether confirming that the stomach is the main site of ghrelin production (Cummings et al., 2002b). These findings suggest that detailed analysis of the cognitive capacity of patients who underwent the gastric bypass surgery would be essential for the clarification of this arising idea.

Cues for Future Research

Ghrelin and cognition

A great number of cognitive tests on laboratory rodents and nonhuman primates are often carried out after food deprivation or fasting, metabolic states that are paralleled by elevated levels of circulating ghrelin. In agreement with this idea, caloric restriction, which results in an increase in the transport of ghrelin across the blood-brain barrier (BBB) (Banks et al., 2008) and in the circulating levels of ghrelin in mice (60% of caloric restriction for 10 days, causing a four-fold increase; Lutter et al., 2008), decreases aging-related deficiencies in cognitive processes in elderly humans (30% caloric resctriction for 3 months; Witte et al., 2009), whilst increasing learning consolidation and facilitating synaptic plasticity in mice [feeding the animals in alternate days for 6–8 months – long-term intermittent fasting diet (L-IFD); Fontán-Lozano et al., 2007].

On the other hand, high-fat and high-glucose diets, which inhibit ghrelin secretion (Beck et al., 2002; Lomenick et al., 2009) and the transport of ghrelin across the BBB (Banks et al., 2008), impair hippocampus-dependent synaptic plasticity and spatial memory (Wu et al., 2003; Stranahan et al., 2008). These models, interfering physiologically with ghrelin circulating levels, would be a great way of validating *in vivo* the assumption that triggering of GHS-R1a-mediated signaling increases the trafficking of AMPARs, thus leading to the potentiation of glutamatergic synaptic transmission and ultimately cognitive enhancement.

Grayson and co-workers compared the effect of caloric restriction and of two different types of bariatric surgery (gastric bypass and gastrectomy) in rats on hippocampal-dependent cognitive function (Grayson et al., 2013). Bariatric surgery is the most effective means of producing weigh loss that is sustained over many years (Sjöström et al., 2004). While gastric bypass was associated, in all behavior paradigms tested (Morris water maze test, 8-arm radial maze task and 4-arm maze task), with an improvement in the performance compared with obese animals, animals subjected to gastrectomy showed an improvement only in the 8-arm radial maze task (Grayson et al., 2013). Unexpectedly, animals subjected to gastrectomy exhibited a significant cognitive deficit when compared with obese animals, when assessed by the Morris water maze test (Grayson et al., 2013). Moreover, they found that 30% of caloric restriction improved behavioral performance in the 8-arm radial maze task (for 8 days) and in the Morris water maze test (for 22 days), comparing with obese animals (Grayson et al., 2013). The authors also measured the circulating levels of ghrelin. They observed the lowest levels in the animals subjected to gastrectomy, and an increase in the animals subjected to caloric restriction (Grayson et al., 2013), in agreement with what have been described for humans subjected to this surgery (Leonetti et al., 2003) and with another protocol of caloric restriction in rodents (Lutter et al., 2008). Unexpectedly, they found an increase of ghrelin circulating levels in animals subjected to gastric bypass, in disagreement with what is described for humans (Cummings et al., 2002b). However, the most exciting result came out when the authors evaluated the correlation between the circulating levels of ghrelin and the performance in the Morris water maze test. Indeed, they found a positive correlation between the behavior performance in the Morris water maze test and ghrelin levels, thus suggesting that high levels of circulating ghrelin are associated with better performances in the Morris water maze test, a hippocampal memory test-dependent. On the other hand, they found that in the experimental condition associated with low levels of ghrelin (gastrectomy) there was a deficit of memory in this behavioral paradigm. Altogether, these observations further support the notion that in metabolic states associated with high ghrelin levels there is a cognitive enhancement.

Despite the observations mentioned above, suggesting that high ghrelin levels induced by caloric restriction are paralleled by cognitive enhancement, Carlini and co-workers using a more severe protocol of caloric restriction in mice (50% for 28 days), found that chronic food restriction, although increasing ghrelin plasma levels, induced memory deficits as assessed by the novel object recognition test (Carlini et al., 2008). This observation is in agreement with the idea that chronic food restriction (like in undernourishment and anorexia) induces impairment of higher brain functions such as learning and memory in mammals (reviewed in Georgieff, 2007). However, they observed that the acute central administration (intracerebroventricular) of ghrelin restores the performance in the animals subjected to this chronic food restriction (Carlini et al., 2008). This result indicates that central exogenous administration of ghrelin ameliorates the behavioral performance in mice subjected to a chronic caloric restriction, which is paralleled by an increase in the plasma ghrelin levels. Thus, despite being present at the periphery, ghrelin is

unable to act centrally. This might happen because it is not being transported across the blood-brain-barrier or because it acts through an indirect mechanism (vagus nerve) that is blocked in this experimental condition. Alternatively, assuming that ghrelin is being transported across the blood brain barrier, and that it reaches the hippocampus (or indirectly vagus nerve is modulating its function), it may be that exacerbated levels of ghrelin for a long period impair synaptic plasticity in the hippocampus, which upon central acute injection of ghrelin is ameliorated. Are other hormones or other brain regions involved? What happens in different species, since ghrelin from different organisms seems to have different ability to cross the blood brain barrier?

The answer to these questions is far from being solved. However, the direct link between the increase in plasma ghrelin levels upon caloric restriction, and the improvement in the hippocampal-dependent memory performance could be probably easily achieved. Submitting rodents to a caloric restriction protocol (60% of caloric restriction for 10 days), which is well described as being able to up-regulate the plasma ghrelin (Lutter et al., 2008), we could start to confirm the efficiency of this type of protocol to induce learning and memory improvement (performing behavioral tests in control and food restricted rodents). These experiments could be repeated when injecting in the hippocampus the ghrelin receptor antagonist or blocking ghrelin in the serum (conditions that would be expected to block the effects of caloric restriction on hippocampal-dependent memory performance).

Alternatively, this could be confirmed using ghrelin receptor knockout animals that are known to display a cognitive deficit. The prediction is that, as opposed to wild-type animals, in these animals cognitive performances are not improved after caloric restriction. If this prediction is confirmed, it would prove the requirement of ghrelin receptor, and hence ghrelin, in this biological paradigm.

Ghrelin and AMPA receptor traffic

After establishing that ghrelin receptor-mediated signaling is required for caloric restriction-induced memory improvement, this physiological context, which triggers the increase of ghrelin levels in the plasma, could be used to prove that GluA1-containing AMPARs synaptic trafficking is required for cognitive enhancement mediated by ghrelin in an *in vivo* perspective. Protein biotinylation could be done using hippocampi from rodents subjected to caloric restriction to assess for an increase in GluA1 synaptic insertion. Additionally, hippocampal postsynaptic density fractions could be prepared from animals subjected to the same paradigm to further identify changes in GluA1 synaptic trafficking upon increase in the circulating ghrelin. Moreover, functional properties of the glutamatergic transmission could be assessed by performing electrophysiological recordings in hippocampal acute slices prepared from animal subjected to this protocol, evaluating changes in field excitatory postsynaptic currents (fEPSC). These assays should be conducted in parallel with untreated animals, and with knockout

animals for ghrelin receptor, to prove unequivocally that the excitatory transmission enhancement observed is dependent on ghrelin receptor-mediated signaling.

To additionally prove that the molecular mechanism here suggested (Figure 1) is the cellular substrate for better performances on memory-dependent behavioral tests when ghrelin is central or peripherally administered in rodents, by repeating this administration, and by performing protein biotinylation, the subunit GluA1 of AMPARs should be increased in surface fractions prepared from hippocampus. Again, this analysis could also be made in hippocampal postsynaptic density fractions, and in parallel using control animals, knockout animals for ghrelin receptor or injecting in the hippocampus the antagonist of ghrelin receptor. A critical assay would be to administrate ghrelin, using a protocol which causes hippocampal-dependent memory enhancement, in knock-in animals expressing the phosphorylation sites of GluA1 Ser^{845} and Ser^{831} mutated to alanine (Lee et al., 2003) and verifying that in these animals administration of ghrelin is unable to produce cognitive enhancement when assessed by hippocampal-dependent behavior tasks. In case the treatment causes an increased memory retention, this would indicate that the mechanism does not depend on an increase of GluA1 phosphorylation.

Here we show that ghrelin has a pivot role in the modulation of excitatory glutamatergic neurotransmission in the hippocampus, strengthening the neurotransmission by inducing AMPARs trafficking, function that we believe may underlie learning and memory formation necessary to satisfy the energetic metabolism. In addition, some emerging observations reveal that this orexigenic factor may modulate other vital physiological functions through its effect on glutamatergic-mediated neurotransmission in other brain regions.

Abizaid and co-workers found that the frequency of action potentials in dopaminergic neurons in the ventral tegmental area (VTA) is increased by ghrelin treatment (Abizaid et al., 2006). However, no effect of ghrelin was observed on frequency of action potentials in slices treated with CNQX (AMPARs antagonist) and AP5 (NMDARs antagonist) (Abizaid et al., 2006), suggesting that ghrelin mediated increment in the frequency of action potentials in dopaminergic neurons requires excitatory inputs to these neurons. Moreover, these authors found that intraperitoneal injection of ghrelin also increased the number of asymmetric excitatory synapses, while symmetric inhibitory contacts were reduced after ghrelin treatment (Abizaid et al., 2006), in aggrement with what have been found in the hippocampus. In correlation with the previous results, they observed that ghrelin treatment significantly increased the frequency of mEPSCs, while it decreased the frequency of mIPSCs (Abizaid et al., 2006). Altogether these observations suggest that VTA is a highly plastic area of the brain, where glutamatergic transmission seems to play a pivot role. This regions has emerged as a target of ghrelin to incentive the motivation for natural rewards such as food (reviewed in Diz-Chaves, 2011, Perelló and Zigman, 2012 and Schellekens et al., 2012).

Moreover, the interaction between ghrelin and glutamatergic system was also implicated in ghrelin-induced feeding behavior in broiler cockerels (Taati et al., 2011). Taati and co-workers found that intracerebroventricular injection of ghrelin, as previously described (Furuse et al., 2001; Saito et al., 2005), strongly inhibits food intake in a dose dependent-manner in chickens (Taati et al., 2011). Moreover, they found that intracerebroventricular injection of ghrelin was attenuated by pretreatment with AP5 and co-administration of glutamate and ghrelin additively enhanced inhibitory effect of ghrelin on food intake (Taati et al., 2011). These results indicate that glutamatergic system is involved in the ghrelin induced regulation of feeding behavior.

Furthermore, Liu and co-workers found that deletion of NMDARs from Agouti-related peptide (AgRP)-expressing neurons in the hypothalamus caused marked reductions in body weight as well as in the *ad libitum* food intake (Liu et al., 2012). They also showed that 24 hours of fasting markedly increased spine number on AgRP neuron dendrites and the frequency of mEPSCs (Liu et al., 2012). Both of these phenotypes were lost in AgRP neurons lacking postsynaptic NMDARs. Ghrelin is known to activate AgRP neurons (Cowley et al., 2003); and so, one interesting possibility is that ghrelin, which is increased by fasting in the plasma, might modulate this effect, similarly with what happens in the hippocampus (Diano et al., 2006; Cuellar and Isokawa, 2011) and VTA (Abizaid et al., 2006), regions where it seems to act as an inducer of spine formation.

Altoghether these observations point to a scenario where ghrelin is having a role in the modulation of glutamatergic transmission throughout the brain. Hence, a detailed analysis of ghrelin´s action on glutamatergic neurotransmission in other brain regions, as presented here for the hippocampus, is clearly being missed.

To conclude, our data suggest that ghrelin-induced enhancement of cognition is likely based on its ability to drive GluA1-containing AMPARs to synapses, a mechanism known to be the molecular basis of memory acquisition and storage in the hippocampus. The following observations support this idea:

i) ghrelin receptors are present at excitatory synapses in the hippocampus;
ii) ghrelin receptor activation promotes the synaptic localization of AMPARs in hippocampal cultures;
iii) ghrelin receptor activation enhances AMPAR-mediated currents, drives the synaptic insertion of GluA1-containing AMPARs in an activity-dependent manner, and enhances long-term potentiation in the hippocampus;
iv) ghrelin receptor activation triggers the phosphorylation of GluA1 and stargazin.

Chapter 6

References

Abizaid, A., Liu, Z.-W., Andrews, Z.B., Shanabrough, M., Borok, E., Elsworth, J.D., Roth, R.H., Sleeman, M.W., Picciotto, M.R., Tschöp, M.H., et al. (2006). Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. The Journal of Clinical Investigation *116*, 3229–3239.

Adan, R.A., Cone, R.D., Burbach, J.P., and Gispen, W.H. (1994). Differential effects of melanocortin peptides on neural melanocortin receptors. Molecular Pharmacology *46*, 1182–1190.

Adesnik, H., Nicoll, R.A., and England, P.M. (2005). Photoinactivation of native AMPA receptors reveals their real-time trafficking. Neuron *48*, 977–985.

Ahmad, M., Polepalli, J.S., Goswami, D., Yang, X., Kaeser-Woo, Y.J., Südhof, T.C., and Malenka, R.C. (2012). Postsynaptic complexin controls AMPA receptor exocytosis during LTP. Neuron *73*, 260–267.

Akman, M.S., Girard, M., O'Brien, L.F., Ho, A.K., and Chik, C.L. (1993). Mechanisms of action of a second generation growth hormone-releasing peptide (Ala-His-D-beta Nal-Ala-Trp-D-Phe-Lys-NH2) in rat anterior pituitary cells. Endocrinology *132*, 1286–1291.

Albarran-Zeckler, R.G., Brantley, A.F., and Smith, R.G. (2012). Growth hormone secretagogue receptor (GHS-R1a) knockout mice exhibit improved spatial memory and deficits in contextual memory. Behavioural Brain Research *232*, 13–19.

Albensi, B.C., Oliver, D.R., Toupin, J., and Odero, G. (2007). Electrical stimulation protocols for hippocampal synaptic plasticity and neuronal hyper-excitability: are they effective or relevant? Experimental Neurology *204*, 1–13.

Aloyo, V.J., Berg, K. a, Clarke, W.P., Spampinato, U., and Harvey, J. a (2010). Inverse agonism at serotonin and cannabinoid receptors. Progress in Molecular Biology and Translational Science *91*, 1-40.

Anderson, L.L., Jeftinija, S., and Scanes, C.G. (2004). Growth hormone secretion: molecular and cellular mechanisms and in vivo approaches. Experimental Biology and Medicine *229*, 291–302.

Andersson, U., Filipsson, K., Abbott, C.R., Woods, A., Smith, K., Bloom, S.R., Carling, D., and Small, C.J. (2004). AMP-activated protein kinase plays a role in the control of food intake. The Journal of Biological Chemistry *279*, 12005–12008.

Andrews, Z.B. (2011). The extra-hypothalamic actions of ghrelin on neuronal function. Trends in Neurosciences *34*, 31–40.

Anggono, V., and Huganir, R.L. (2012). Regulation of AMPA receptor trafficking and synaptic plasticity. Current Opinion in Neurobiology *22*, 461–469.

Arendt, K.L., Royo, M., Fernández-Monreal, M., Knafo, S., Petrok, C.N., Martens, J.R., and Esteban, J. a (2010). PIP3 controls synaptic function by maintaining AMPA receptor clustering at the postsynaptic membrane. Nature Neuroscience *13*, 36–44.

Ariyasu, H., Takaya, K., Tagami, T., Ogawa, Y., Hosoda, K., Akamizu, T., Suda, M., Koh, T., Natsui, K., Toyooka, S., et al. (2001). Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. The Journal of Clinical Endocrinology and Metabolism *86*, 4753–4758.

Arvat, E., Maccario, M., Di Vito, L., Broglio, F., Benso, A., Gottero, C., Papotti, M., Muccioli, G., Dieguez, C., Casanueva, F.F., et al. (2001). Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. The Journal of Clinical Endocrinology and Metabolism *86*, 1169–1174.

Arvat, E., Di Vito, L., Broglio, F., Papotti, M., Muccioli, G., Dieguez, C., Casanueva, F.F., Deghenghi, R., Camanni, F., and Ghigo, E. (2000). Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. Journal of Endocrinological Investigation *23*, 493–495.

Asakawa, A., Inui, A., Kaga, T., Katsuura, G., Fujimiya, M., Fujino, M.A., and Kasuga, M. (2003). Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. Gut *52*, 947–952.

Asakawa, A., Inui, A., Kaga, T., Yuzuriha, H., Nagata, T., Ueno, N., Makino, S., Fujimiya, M., Niijima, A., Fujino, M.A., et al. (2001a). Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology *120*, 337–345.

Asakawa, A., Inui, A., T, K., Yuzuriha, H., Nagata, T., Fujimiya, M., Katsuura, G., Makino, S., Fujino, M., and Kasuga, M. (2001b). A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. Neuroendocrinology *74*, 143–147.

Asrican, B., Lisman, J., and Otmakhov, N. (2007). Synaptic strength of individual spines correlates with bound Ca2+-calmodulin-dependent kinase II. The Journal of Neuroscience *27*, 14007–14011.

Atcha, Z., Chen, W.-S., Ong, A.B., Wong, F.-K., Neo, A., Browne, E.R., Witherington, J., and Pemberton, D.J. (2009). Cognitive enhancing effects of ghrelin receptor agonists. Psychopharmacology *206*, 415–427.

Autelitano, D.J., Rajic, A., Smith, A.I., Berndt, M.C., Ilag, L.L., and Vadas, M. (2006). The cryptome: a subset of the proteome, comprising cryptic peptides with distinct bioactivities. Drug Discovery Today *11*, 306–314.

Bagnasco, M., Kalra, P.S., and Kalra, S.P. (2002). Ghrelin and leptin pulse discharge in fed and fasted rats. Endocrinology *143*, 726–729.

Banke, T.G., Bowie, D., Lee, H., Huganir, R.L., Schousboe, A., and Traynelis, S.F. (2000). Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. The Journal of Neuroscience *20*, 89–102.

Banks, W.A., Burney, B.O., and Robinson, S.M. (2008). Effects of triglycerides, obesity, and starvation on ghrelin transport across the blood-brain barrier. Peptides *29*, 2061–2065.

Banks, W.A., Tschöp, M., Robinson, S.M., and Heiman, M.L. (2002). Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. The Journal of Pharmacology and Experimental Therapeutics *302*, 822–827.

Barim, A.O., Aydin, S., Colak, R., Dag, E., Deniz, O., and Sahin, I. (2009). Ghrelin, paraoxonase and arylesterase levels in depressive patients before and after citalopram treatment. Clinical Biochemistry *42*, 1076–1081.

Barkan, A.L., Dimaraki, E. V, Jessup, S.K., Symons, K. V, Ermolenko, M., and Jaffe, C.A. (2003). Ghrelin secretion in humans is sexually dimorphic, suppressed by somatostatin, and not affected by the ambient growth hormone levels. The Journal of Clinical Endocrinology and Metabolism *88*, 2180–2184.

Barreiro, M.L., Gaytán, F., Caminos, J.E., Pinilla, L., Casanueva, F.F., Aguilar, E., Diéguez, C., and Tena-Sempere, M. (2002). Cellular location and hormonal regulation of ghrelin expression in rat testis. Biology of Reproduction *67*, 1768–1776.

Barria, A., Derkach, V., and Soderling, T. (1997). Identification of the Ca2+/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor. The Journal of Biological Chemistry *272*, 32727–32730.

Baskin, D.G., Breininger, J.F., and Schwartz, M.W. (1999). Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. Diabetes *48*, 828–833.

Bats, C., Groc, L., and Choquet, D. (2007). The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. Neuron *53*, 719–734.

Bayer, K.U., De Koninck, P., Leonard, A.S., Hell, J.W., and Schulman, H. (2001). Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature *411*, 801–805.

Bayer, K.U., LeBel, E., McDonald, G.L., O'Leary, H., Schulman, H., and De Koninck, P. (2006). Transition from reversible to persistent binding of CaMKII to postsynaptic sites and NR2B. The Journal of Neuroscience *26*, 1164–1174.

Beattie, E.C., Carroll, R.C., Yu, X., Morishita, W., Yasuda, H., Von Zastrow, M., and Malenka, R.C. (2000). Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. Nature Neuroscience *3*, 1291–1300.

Beck, B., Musse, N., and Stricker-Krongrad, A. (2002). Ghrelin, macronutrient intake and dietary preferences in long-evans rats. Biochemical and Biophysical Research Communications *292*, 1031–1035.

Bednarek, M.A., Feighner, S.D., Pong, S.S., McKee, K.K., Hreniuk, D.L., Silva, M. V, Warren, V.A., Howard, A.D., Van Der Ploeg, L.H., and Heck, J. V (2000). Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. Journal of Medicinal Chemistry *43*, 4370–4376.

Bellone, S., Rapa, A., Vivenza, D., Castellino, N., Petri, A., Bellone, J., Me, E., Broglio, F., Prodam, F., Ghigo, E., et al. (2002). Circulating ghrelin levels as function of gender, pubertal status and adiposity in childhood. Journal of Endocrinological Investigation *25*, RC13–5.

Benoit, S., Schwartz, M., Baskin, D., Woods, S.C., and Seeley, R.J. (2000). CNS melanocortin system involvement in the regulation of food intake. Hormones and Behavior *37*, 299–305.

Berrout, L., and Isokawa, M. (2012). Ghrelin promotes reorganization of dendritic spines in cultured rat hippocampal slices. Neuroscience Letters *516*, 280–284.

Biebermann, H., Castañeda, T.R., Van Landeghem, F., Von Deimling, A., Escher, F., Brabant, G., Hebebrand, J., Hinney, A., Tschöp, M.H., Grüters, A., et al. (2006). A role for beta-melanocyte-stimulating hormone in human body-weight regulation. Cell Metabolism *3*, 141–146.

Black, D.L. (2003). Mechanisms of alternative pre-messenger RNA splicing. Annual Review of Biochemistry *72*, 291–336.

Blake, A.D., and Smith, R.G. (1991). Desensitization studies using perifused rat pituitary cells show that growth hormone-releasing hormone and His-D-Trp-Ala-Trp-D-Phe-Lys-NH2 stimulate growth hormone release through distinct receptor sites. The Journal of Endocrinology *129*, 11–19.

Bliss, T. V, and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature *361*, 31–39.

Bliss, T. V, and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. The Journal of Physiology *232*, 331–356.

Bockaert, J., and Pin, J.P. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. The EMBO Journal *18*, 1723–1729.

Bodart, V., Bouchard, J.F., McNicoll, N., Escher, E., Carrière, P., Ghigo, E., Sejlitz, T., Sirois, M.G., Lamontagne, D., and Ong, H. (1999). Identification and characterization of a new growth hormone-releasing peptide receptor in the heart. Circulation Research *85*, 796–802.

Boehm, J., Kang, M.-G., Johnson, R.C., Esteban, J., Huganir, R.L., and Malinow, R. (2006). Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. Neuron *51*, 213–225.

Böhme, G.A., Bon, C., Lemaire, M., Reibaud, M., Piot, O., Stutzmann, J.M., Doble, A., and Blanchard, J.C. (1993). Altered synaptic plasticity and memory formation in nitric oxide synthase inhibitor-treated rats. Proceedings of the National Academy of Sciences of the United States of America *90*, 9191–9194.

Bond, R.A., and Ijzerman, A.P. (2006). Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. Trends in Pharmacological Sciences *27*, 92–96.

Borgdorff, A.J., and Choquet, D. (2002). Regulation of AMPA receptor lateral movements. Nature *417*, 649–653.

Boston, B.A., Blaydon, K.M., Varnerin, J., and Cone, R.D. (1997). Independent and additive effects of central POMC and leptin pathways on murine obesity. Science *278*, 1641–1644.

Boulter, J., Hollmann, M., O'Shea-Greenfield, A., Hartley, M., Deneris, E., Maron, C., and Heinemann, S. (1990). Molecular cloning and functional expression of glutamate receptor subunit genes. Science *249*, 1033–1037.

Bowers, C.Y., Momany, F., Reynolds, G.A., Chang, D., Hong, A., and Chang, K. (1980). Structure-activity relationships of a synthetic pentapeptide that specifically releases growth hormone in vitro. Endocrinology *106*, 663–667.

Bowers, C.Y., Momany, F.A., Reynolds, G.A., and Hong, A. (1984). On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology *114*, 1537–1545.

Bowers, C.Y., Reynolds, G.A., Durham, D., Barrera, C.M., Pezzoli, S.S., and Thorner, M.O. (1990). Growth hormone (GH)-releasing peptide stimulates GH release in normal men and acts synergistically with GH-releasing hormone. The Journal of Clinical Endocrinology and Metabolism *70*, 975–982.

Bredt, D.S., and Nicoll, R.A. (2003). AMPA receptor trafficking at excitatory synapses. Neuron *40*, 361–379.

Callahan, H.S., Cummings, D.E., Pepe, M.S., Breen, P.A., Matthys, C.C., and Weigle, D.S. (2004). Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load but does not predict intermeal interval in humans. The Journal of Clinical Endocrinology and Metabolism *89*, 1319–1324.

Camanni, F., Ghigo, E., and Arvat, E. (1998). Growth hormone-releasing peptides and their analogs. Frontiers in Neuroendocrinology *19*, 47–72.

Camiña, J.P. (2006). Cell biology of the ghrelin receptor. Journal of Neuroendocrinology *18*, 65–76.

Caminos, J.E., Tena-Sempere, M., Gaytán, F., Sanchez-Criado, J.E., Barreiro, M.L., Nogueiras, R., Casanueva, F.F., Aguilar, E., and Diéguez, C. (2003). Expression of ghrelin in the cyclic and pregnant rat ovary. Endocrinology *144*, 1594–1602.

Carling, D. (2005). AMP-activated protein kinase: balancing the scales. Biochimie *87*, 87–91.

Carlini, V.P., Gaydou, R.C., Schiöth, H.B., and De Barioglio, S.R. (2007). Selective serotonin reuptake inhibitor (fluoxetine) decreases the effects of ghrelin on memory retention and food intake. Regulatory Peptides *140*, 65–73.

Carlini, V.P., Ghersi, M., Schiöth, H.B., and De Barioglio, S.R. (2010a). Ghrelin and memory: differential effects on acquisition and retrieval. Peptides *31*, 1190–1193.

Carlini, V.P., Martini, A.C., Schiöth, H.B., Ruiz, R.D., Fiol de Cuneo, M., and De Barioglio, S.R. (2008). Decreased memory for novel object recognition in chronically food-restricted mice is reversed by acute ghrelin administration. Neuroscience *153*, 929–934.

Carlini, V.P., Monzón, M.E., Varas, M.M., Cragnolini, A.B., Schiöth, H.B., Scimonelli, T.N., and De Barioglio, S.R. (2002). Ghrelin increases anxiety-like behavior and memory retention in rats. Biochemical and Biophysical Research Communications *299*, 739–743.

Carlini, V.P., Perez, M.F., Salde, E., Schiöth, H.B., Ramirez, O.A., and De Barioglio, S.R. (2010b). Ghrelin induced memory facilitation implicates nitric oxide synthase activation and decrease in the threshold to promote LTP in hippocampal dentate gyrus. Physiology & Behavior *101*, 117–123.

Carlini, V.P., Varas, M.M., Cragnolini, A.B., Schiöth, H.B., Scimonelli, T.N., and De Barioglio, S.R. (2004). Differential role of the hippocampus, amygdala, and dorsal raphe nucleus in regulating feeding, memory, and anxiety-like behavioral responses to ghrelin. Biochemical and Biophysical Research Communications *313*, 635–641.

Carvajal, P., Carlini, V.P., Schiöth, H.B., De Barioglio, S.R., and Salvatierra, N.A. (2009). Central ghrelin increases anxiety in the Open Field test and impairs retention memory in a passive avoidance task in neonatal chicks. Neurobiology of Learning and Memory *91*, 402–407.

Carvalho, A.L., Kameyama, K., and Huganir, R.L. (1999). Characterization of phosphorylation sites on the glutamate receptor 4 subunit of the AMPA receptors. The Journal of Neuroscience *19*, 4748–4754.

Chan, J.L., Bullen, J., Lee, J.H., Yiannakouris, N., and Mantzoros, C.S. (2004). Ghrelin levels are not regulated by recombinant leptin administration and/or three days of fasting in healthy subjects. The Journal of Clinical Endocrinology and Metabolism *89*, 335–343.

Chanoine, J.-P., and Wong, A.C.K. (2004). Ghrelin gene expression is markedly higher in fetal pancreas compared with fetal stomach: effect of maternal fasting. Endocrinology *145*, 3813–3820.

Chapman, I.M., Bach, M.A., Van Cauter, E., Farmer, M., Krupa, D., Taylor, A.M., Schilling, L.M., Cole, K.Y., Skiles, E.H., Pezzoli, S.S., et al. (1996). Stimulation of the growth hormone (GH)-insulin-like growth factor I axis by daily oral administration of a GH secretogogue (MK-677) in healthy elderly subjects. The Journal of Clinical Endocrinology and Metabolism *81*, 4249–4257.

Chen, C.-Y., Asakawa, A., Fujimiya, M., Lee, S.-D., and Inui, A. (2009). Ghrelin gene products and the regulation of food intake and gut motility. Pharmacological Reviews *61*, 430–481.

Chen, L., Chetkovich, D.M., Petralia, R.S., Sweeney, N.T., Kawasaki, Y., Wenthold, R.J., Bredt, D.S., and Nicoll, R.A. (2000). Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature *408*, 936–943.

Chen, L., Xing, T., Wang, M., Miao, Y., Tang, M., Chen, J., Li, G., and Ruan, D.-Y. (2011). Local infusion of ghrelin enhanced hippocampal synaptic plasticity and spatial memory through activation of phosphoinositide 3-kinase in the dentate gyrus of adult rats. The European Journal of Neuroscience *33*, 266–275.

Cheng, K., Chan, W.W., Butler, B., Barreto, A., and Smith, R.G. (1991). Evidence for a role of protein kinase-C in His-D-Trp-Ala-Trp-D-Phe-Lys-NH2-induced growth hormone release from rat primary pituitary cells. Endocrinology *129*, 3337–3342.

Cheng, K., Chan, W.W., Butler, B., Wei, L., Schoen, W.R., Wyvratt, M.J., Fisher, M.H., and Smith, R.G. (1993). Stimulation of growth hormone release from rat primary pituitary cells by L-692,429, a novel non-peptidyl GH secretagogue. Endocrinology *132*, 2729–2731.

Chow, K.B.S., Sun, J., Chu, K.M., Tai Cheung, W., Cheng, C.H.K., and Wise, H. (2012). The truncated ghrelin receptor polypeptide (GHS-R1b) is localized in the endoplasmic reticulum where it forms heterodimers with ghrelin receptors (GHS-R1a) to attenuate their cell surface expression. Molecular and Cellular Endocrinology *348*, 247–254.

Chronwall, B.M., Chase, T.N., and O'Donohue, T.L. (1984). Coexistence of neuropeptide Y and somatostatin in rat and human cortical and rat hypothalamic neurons. Neuroscience Letters *52*, 213–217.

Chung, H.J., Xia, J., Scannevin, R.H., Zhang, X., and Huganir, R.L. (2000). Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. The Journal of Neuroscience *20*, 7258–7267.

Civelli, O. (1998). Functional genomics: the search for novel neurotransmitters and neuropeptides. FEBS Letters *430*, 55–58.

Civelli, O., Nothacker, H.P., Saito, Y., Wang, Z., Lin, S.H., and Reinscheid, R.K. (2001). Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. Trends in Neurosciences *24*, 230–237.

Clark, J.T., Kalra, P.S., Crowley, W.R., and Kalra, S.P. (1984). Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. Endocrinology *115*, 427–429.

Collingridge, G.L., Isaac, J.T.R., and Wang, Y.T. (2004). Receptor trafficking and synaptic plasticity. Nature Reviews Neuroscience *5*, 952–962.

Cone, R.D. (2005). Anatomy and regulation of the central melanocortin system. Nature Neuroscience *8*, 571–578.

Cone, R.D., Cowley, M.A., Butler, A.A., Fan, W., Marks, D.L., and Low, M.J. (2001). The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. International Journal of Obesity and Related Metabolic Disorders *25 Suppl 5*, S63–7.

Contractor, A., Mulle, C., and Swanson, G.T. (2011). Kainate receptors coming of age: milestones of two decades of research. Trends in Neurosciences *34*, 154–163.

Corradetti, R., Ballerini, L., Pugliese, A.M., and Pepeu, G. (1992). Serotonin blocks the long-term potentiation induced by primed burst stimulation in the CA1 region of rat hippocampal slices. Neuroscience *46*, 511–518.

Correia, S.S., Duarte, C.B., Faro, C.J., Pires, E.V., and Carvalho, A.L. (2003). Protein kinase C gamma associates directly with the GluR4 alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor subunit. Effect on receptor phosphorylation. The Journal of Biological Chemistry *278*, 6307–6313.

Coulie, B.J., and Miller, L.J. (2001). Identification of motilin-related peptide. Gastroenterology *120*, 588–589.

Cowley, M.A., Smith, R.G., Diano, S., Tschöp, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L., et al. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron *37*, 649–661.

Cuellar, J.N., and Isokawa, M. (2011). Ghrelin-induced activation of cAMP signal transduction and its negative regulation by endocannabinoids in the hippocampus. Neuropharmacology *60*, 842–851.

Cummings, D.E., Clement, K., Purnell, J.Q., Vaisse, C., Foster, K.E., Frayo, R.S., Schwartz, M.W., Basdevant, A., and Weigle, D.S. (2002a). Elevated plasma ghrelin levels in Prader Willi syndrome. Nature Medicine *8*, 643–644.

Cummings, D.E., Frayo, R.S., Marmonier, C., Aubert, R., and Chapelot, D. (2004). Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. American Journal of Physiology. Endocrinology and Metabolism *287*, E297–304.

Cummings, D.E., Overduin, J., Shannon, M.H., and Foster-Schubert, K.E. (2005). Hormonal mechanisms of weight loss and diabetes resolution after bariatric surgery. Surgery for Obesity and Related Diseases *1*, 358–368.

Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E., and Weigle, D.S. (2001). A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes *50*, 1714–1719.

Cummings, D.E., Weigle, D.S., Frayo, R.S., Breen, P. a, Ma, M.K., Dellinger, E.P., and Purnell, J.Q. (2002b). Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. The New England Journal of Medicine *346*, 1623–1630.

Cuntz, U., Frühauf, E., Wawarta, R., Tschöp, M., Folwaczny, C., Riepl, R., Lehnert, P., Fichter, M., and Otto, B. (2002). A role for the novel weight-regulating hormone ghrelin in anorexia nervosa. American Clinical Laboratory *21*, 22–23.

Date, Y., Kojima, M., Hosoda, H., Sawaguchi, A., Mondal, M.S., Suganuma, T., Matsukura, S., Kangawa, K., and Nakazato, M. (2000a). Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology *141*, 4255–4261.

Date, Y., Murakami, N., Kojima, M., Kuroiwa, T., Matsukura, S., Kangawa, K., and Nakazato, M. (2000b). Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. Biochemical and Biophysical Research Communications *275*, 477–480.

Date, Y., Murakami, N., Toshinai, K., Matsukura, S., Niijima, A., Matsuo, H., Kangawa, K., and Nakazato, M. (2002a). The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology *123*, 1120–1128.

Date, Y., Nakazato, M., Hashiguchi, S., Dezaki, K., Mondal, M.S., Hosoda, H., Kojima, M., Kangawa, K., Arima, T., Matsuo, H., et al. (2002b). Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. Diabetes *51*, 124–129.

Davenport, A.P., Bonner, T.I., Foord, S.M., Harmar, A.J., Neubig, R.R., Pin, J.-P., Spedding, M., Kojima, M., and Kangawa, K. (2005). International Union of Pharmacology. LVI. Ghrelin receptor nomenclature, distribution, and function. Pharmacological Reviews *57*, 541–546.

Davis, J.F., Choi, D.L., Clegg, D.J., and Benoit, S.C. (2011). Signaling through the ghrelin receptor modulates hippocampal function and meal anticipation in mice. Physiology & Behavior *103*, 39–43.

Daw, M.I., Chittajallu, R., Bortolotto, Z.A., Dev, K.K., Duprat, F., Henley, J.M., Collingridge, G.L., and Isaac, J.T. (2000). PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. Neuron *28*, 873–886.

Deghenghi, R., Cananzi, M.M., Torsello, A., Battisti, C., Muller, E.E., and Locatelli, V. (1994). GH-releasing activity of Hexarelin, a new growth hormone releasing peptide, in infant and adult rats. Life Sciences *54*, 1321–1328.

Delgado, J.Y., Coba, M., Anderson, C.N.G., Thompson, K.R., Gray, E.E., Heusner, C.L., Martin, K.C., Grant, S.G.N., and O'Dell, T.J. (2007). NMDA receptor activation dephosphorylates AMPA receptor glutamate receptor 1 subunits at threonine 840. The Journal of Neuroscience□*27*, 13210–13221.

Delhanty, P.J.D., Neggers, S.J., and Van der Lely, A.J. (2012). Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. European Journal of Endocrinology *167*, 601–608.

Derkach, V., Barria, A., and Soderling, T.R. (1999). Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proceedings of the National Academy of Sciences of the United States of America *96*, 3269–3274.

Derkach, V.A., Oh, M.C., Guire, E.S., and Soderling, T.R. (2007). Regulatory mechanisms of AMPA receptors in synaptic plasticity. Nature Reviews Neuroscience *8*, 101–113.

Diano, S., Farr, S. a, Benoit, S.C., McNay, E.C., Da Silva, I., Horvath, B., Gaskin, F.S., Nonaka, N., Jaeger, L.B., Banks, W. a, et al. (2006). Ghrelin controls hippocampal spine synapse density and memory performance. Nature Neuroscience *9*, 381–388.

Dingledine, R., Borges, K., Bowie, D., and Traynelis, S.F. (1999). The glutamate receptor ion channels. Pharmacological Reviews *51*, 7–61.

Diz-Chaves, Y. (2011). Ghrelin, appetite regulation, and food reward: interaction with chronic stress. International Journal of Peptides *2011*, 898450.

Dornonville de la Cour, C., Björkqvist, M., Sandvik, A.K., Bakke, I., Zhao, C.M., Chen, D., and Håkanson, R. (2001). A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. Regulatory Peptides *99*, 141–150.

Dragoi, G., Harris, K.D., and Buzsáki, G. (2003). Place representation within hippocampal networks is modified by long-term potentiation. Neuron *39*, 843–853.

Druce, M., and Bloom, S.R. (2003). Central regulators of food intake. Current Opinion in Clinical Nutrition and Metabolic Care *6*, 361–367.

Druce, M.R., Wren, A.M., Park, A.J., Milton, J.E., Patterson, M., Frost, G., Ghatei, M.A., Small, C., and Bloom, S.R. (2005). Ghrelin increases food intake in obese as well as lean subjects. International Journal of Obesity (2005) *29*, 1130–1136.

Durand, G.M., Kovalchuk, Y., and Konnerth, A. (1996). Long-term potentiation and functional synapse induction in developing hippocampus. Nature *381*, 71–75.

Egecioglu, E., Jerlhag, E., Salomé, N., Skibicka, K.P., Haage, D., Bohlooly-Y, M., Andersson, D., Bjursell, M., Perrissoud, D., Engel, J.A., et al. (2010). Ghrelin increases intake of rewarding food in rodents. Addiction Biology *15*, 304–311.

Egecioglu, E., Skibicka, K.P., Hansson, C., Alvarez-Crespo, M., Friberg, P.A., Jerlhag, E., Engel, J.A., and Dickson, S.L. (2011). Hedonic and incentive signals for body weight control. Reviews in Endocrine & Metabolic Disorders *12*, 141–151.

Ehlers, M.D. (2000). Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. Neuron *28*, 511–525.

Elphick, M.R., and Egertová, M. (2001). The neurobiology and evolution of cannabinoid signalling. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences *356*, 381–408.

Erreger, K., Chen, P.E., Wyllie, D.J.A., and Traynelis, S.F. (2004). Glutamate receptor gating. Critical Reviews in Neurobiology *16*, 187–224.

Esler, W.P., Rudolph, J., Claus, T.H., Tang, W., Barucci, N., Brown, S.-E., Bullock, W., Daly, M., Decarr, L., Li, Y., et al. (2007). Small-molecule ghrelin receptor antagonists improve glucose tolerance, suppress appetite, and promote weight loss. Endocrinology *148*, 5175–5185.

Esteban, J. A., Shi, S.-H., Wilson, C., Nuriya, M., Huganir, R.L., and Malinow, R. (2003). PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. Nature Neuroscience *6*, 136–143.

Esteban, J.A. (2003). AMPA receptor trafficking: a road map for synaptic plasticity. Molecular Interventions *3*, 375–385.

Falls, H.D., Dayton, B.D., Fry, D.G., Ogiela, C.A., Schaefer, V.G., Brodjian, S., Reilly, R.M., Collins, C.A., and Kaszubska, W. (2006). Characterization of ghrelin receptor activity in a rat pituitary cell line RC-4B/C. Journal of Molecular Endocrinology *37*, 51–62.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J., and Cone, R.D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature *385*, 165–168.

Faulconbridge, L.F., Cummings, D.E., Kaplan, J.M., and Grill, H.J. (2003). Hyperphagic effects of brainstem ghrelin administration. Diabetes *52*, 2260–2265.

Feighner, S.D., Tan, C.P., McKee, K.K., Palyha, O.C., Hreniuk, D.L., Pong, S.S., Austin, C.P., Figueroa, D., MacNeil, D., Cascieri, M.A., et al. (1999). Receptor for motilin identified in the human gastrointestinal system. Science *284*, 2184–2188.

Finger, B.C., Dinan, T.G., and Cryan, J.F. (2011a). Behavioral satiety sequence in a genetic mouse model of obesity: effects of ghrelin receptor ligands. Behavioural Pharmacology *22*, 624–632.

Finger, B.C., Schellekens, H., Dinan, T.G., and Cryan, J.F. (2011b). Is there altered sensitivity to ghrelin-receptor ligands in leptin-deficient mice?: importance of satiety state and time of day. Psychopharmacology *216*, 421–429.

Folwaczny, C., Chang, J.K., and Tschöp, M. (2001). Ghrelin and motilin: two sides of one coin? European Journal of Endocrinology *144*, R1–3.

Fontán-Lozano, A., Sáez-Cassanelli, J.L., Inda, M.C., De los Santos-Arteaga, M., Sierra-Domínguez, S.A., López-Lluch, G., Delgado-García, J.M., and Carrión, A.M. (2007). Caloric restriction increases learning consolidation and facilitates synaptic plasticity through mechanisms dependent on NR2B subunits of the NMDA receptor. The Journal of Neuroscience *27*, 10185–10195.

Frago, L.M., Baquedano, E., Argente, J., and Chowen, J.A. (2011). Neuroprotective actions of ghrelin and growth hormone secretagogues. Frontiers in Molecular Neuroscience *4*, 23.

Friedman, J.M. (2002). The function of leptin in nutrition, weight, and physiology. Nutrition Reviews *60*, S1–14.

Furuse, M., Tachibana, T., Ohgushi, A., Ando, R., Yoshimatsu, T., and Denbow, D.M. (2001). Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neuroscience Letters *301*, 123–126.

Gähwiler, B.H., Capogna, M., Debanne, D., McKinney, R.A., and Thompson, S.M. (1997). Organotypic slice cultures: a technique has come of age. Trends in Neurosciences *20*, 471–477.

Gao, Q., and Horvath, T.L. (2007). Neurobiology of feeding and energy expenditure. Annual Review of Neuroscience *30*, 367–398.

Garcia, J.M., Cata, J.P., Dougherty, P.M., and Smith, R.G. (2008). Ghrelin prevents cisplatin-induced mechanical hyperalgesia and cachexia. Endocrinology *149*, 455–460.

Garg, A. (2007). The ongoing saga of obestatin: is it a hormone? The Journal of Clinical Endocrinology and Metabolism *92*, 3396–3398.

Gauna, C., Uitterlinden, P., Kramer, P., Kiewiet, R.M., Janssen, J.A.M.J.L., Delhanty, P.J.D., Van Aken, M.O., Ghigo, E., Hofland, L.J., Themmen, A.P.N., et al. (2007). Intravenous glucose administration in fasting rats has differential effects on acylated and unacylated ghrelin in the portal and systemic circulation: a comparison between portal and peripheral concentrations in anesthetized rats. Endocrinology *148*, 5278–5287.

Gaytan, F., Barreiro, M.L., Chopin, L.K., Herington, A.C., Morales, C., Pinilla, L., Casanueva, F.F., Aguilar, E., Diéguez, C., and Tena-Sempere, M. (2003). Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary. The Journal of Clinical Endocrinology and Metabolism *88*, 879–887.

Geiger, J.R., Melcher, T., Koh, D.S., Sakmann, B., Seeburg, P.H., Jonas, P., and Monyer, H. (1995). Relative abundance of subunit mRNAs determines gating and Ca2+ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. Neuron *15*, 193–204.

Geisler, S., and Wise, R.A. (2008). Functional implications of glutamatergic projections to the ventral tegmental area. Reviews in the Neurosciences *19*, 227–244.

Geloneze, B., Tambascia, M.A., Pilla, V.F., Geloneze, S.R., Repetto, E.M., and Pareja, J.C. (2003). Ghrelin: a gut-brain hormone: effect of gastric bypass surgery. Obesity Surgery *13*, 17–22.

Georgieff, M.K. (2007). Nutrition and the developing brain: nutrient priorities and measurement. The American Journal of Clinical Nutrition *85*, 614S–620S.

Ghersi, M.S., Casas, S.M., Escudero, C., Carlini, V.P., Buteler, F., Cabrera, R.J., Schiöth, H.B., and De Barioglio, S.R. (2011). Ghrelin inhibited serotonin release from hippocampal slices. Peptides *32*, 2367–2371.

Gilg, S., and Lutz, T.A. (2006). The orexigenic effect of peripheral ghrelin differs between rats of different age and with different baseline food intake, and it may in part be mediated by the area postrema. Physiology & Behavior *87*, 353–359.

Gnanapavan, S., Kola, B., Bustin, S.A., Morris, D.G., McGee, P., Fairclough, P., Bhattacharya, S., Carpenter, R., Grossman, A.B., and Korbonits, M. (2002). The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. The Journal of Clinical Endocrinology and Metabolism *87*, 2988.

Gomes, A.R., Correia, S.S., Esteban, J.A., Duarte, C.B., and Carvalho, A.L. (2007). PKC anchoring to GluR4 AMPA receptor subunit modulates PKC-driven receptor phosphorylation and surface expression. Traffic *8*, 259–269.

Gomes, A.R., Cunha, P., Nuriya, M., Faro, C.J., Huganir, R.L., Pires, E. V, Carvalho, A.L., and Duarte, C.B. (2004). Metabotropic glutamate and dopamine receptors co-regulate AMPA receptor activity through PKA in cultured chick retinal neurones: effect on GluR4 phosphorylation and surface expression. Journal of Neurochemistry *90*, 673–682.

Goshadrou, F., Kermani, M., Ronaghi, A., and Sajjadi, S. (2013). The effect of ghrelin on MK-801 induced memory impairment in rats. Peptides *44*, 60–65.

Gourcerol, G., Million, M., Adelson, D.W., Wang, Y., Wang, L., Rivier, J., St-Pierre, D.H., and Taché, Y. (2006). Lack of interaction between peripheral injection of CCK and obestatin in the regulation of gastric satiety signaling in rodents. Peptides *27*, 2811–2819.

Gower, A.J., and Lamberty, Y. (1993). The aged mouse as a model of cognitive decline with special emphasis on studies in NMRI mice. Behavioural Brain Research *57*, 163–173.

Granger, A.J., Shi, Y., Lu, W., Cerpas, M., and Nicoll, R.A. (2013). LTP requires a reserve pool of glutamate receptors independent of subunit type. Nature *493*, 495–500.

GRAY, E.G. (1959). Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. Journal of Anatomy *93*, 420–433.

Grayson, B.E., Fitzgerald, M.F., Hakala-Finch, A.P., Ferris, V.M., Begg, D.P., Tong, J., Woods, S.C., Seeley, R.J., Davidson, T.L., and Benoit, S.C. (2013). Improvements in Hippocampal-Dependent memory and Microglial-Infiltration with calorie restriction and gastric bypass surgery but not with vertical sleeve gastrectomy. International Journal of Obesity (2005).

Greger, I.H., Khatri, L., Kong, X., and Ziff, E.B. (2003). AMPA receptor tetramerization is mediated by Q/R editing. Neuron *40*, 763–774.

Greger, I.H., Khatri, L., and Ziff, E.B. (2002). RNA editing at arg607 controls AMPA receptor exit from the endoplasmic reticulum. Neuron *34*, 759–772.

Gruart, A., Muñoz, M.D., and Delgado-García, J.M. (2006). Involvement of the CA3-CA1 synapse in the acquisition of associative learning in behaving mice. The Journal of Neuroscience□*26*, 1077–1087.

Gualillo, O., Caminos, J., Blanco, M., Garcìa-Caballero, T., Kojima, M., Kangawa, K., Dieguez, C., and Casanueva, F. (2001). Ghrelin, a novel placental-derived hormone. Endocrinology *142*, 788–794.

Guan, X.M., Yu, H., Palyha, O.C., McKee, K.K., Feighner, S.D., Sirinathsinghji, D.J., Smith, R.G., Van der Ploeg, L.H., and Howard, A.D. (1997). Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Research. Molecular Brain Research *48*, 23–29.

Gutierrez, J.A., Solenberg, P.J., Perkins, D.R., Willency, J.A., Knierman, M.D., Jin, Z., Witcher, D.R., Luo, S., Onyia, J.E., and Hale, J.E. (2008). Ghrelin octanoylation mediated by an orphan lipid transferase. Proceedings of the National Academy of Sciences of the United States of America *105*, 6320–6325.

Hansen, T.K., Dall, R., Hosoda, H., Kojima, M., Kangawa, K., Christiansen, J.S., and Jørgensen, J.O.L. (2002). Weight loss increases circulating levels of ghrelin in human obesity. Clinical Endocrinology *56*, 203–206.

Hansson, C., Haage, D., Taube, M., Egecioglu, E., Salomé, N., and Dickson, S.L. (2011). Central administration of ghrelin alters emotional responses in rats: behavioural, electrophysiological and molecular evidence. Neuroscience *180*, 201–211.

Haqq, A.M., Farooqi, I.S., O'Rahilly, S., Stadler, D.D., Rosenfeld, R.G., Pratt, K.L., LaFranchi, S.H., and Purnell, J.Q. (2003). Serum ghrelin levels are inversely correlated with body mass index, age, and insulin concentrations in normal children and are markedly increased in Prader-Willi syndrome. The Journal of Clinical Endocrinology and Metabolism *88*, 174–178.

Harrold, J.A., Dovey, T., Cai, X.-J., Halford, J.C.G., and Pinkney, J. (2008). Autoradiographic analysis of ghrelin receptors in the rat hypothalamus. Brain Research *1196*, 59–64.

Hattori, N., Saito, T., Yagyu, T., Jiang, B.H., Kitagawa, K., and Inagaki, C. (2001). GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils. The Journal of Clinical Endocrinology and Metabolism *86*, 4284–4291.

Häusser, M., Spruston, N., and Stuart, G.J. (2000). Diversity and dynamics of dendritic signaling. Science *290*, 739–744.

Hayashi, T., and Huganir, R.L. (2004). Tyrosine phosphorylation and regulation of the AMPA receptor by SRC family tyrosine kinases. The Journal of Neuroscience□*24*, 6152–6160.

Hayashi, T., Rumbaugh, G., and Huganir, R.L. (2005). Differential regulation of AMPA receptor subunit trafficking by palmitoylation of two distinct sites. Neuron *47*, 709–723.

Hayashi, Y., Shi, S.H., Esteban, J.A., Piccini, A., Poncer, J.C., and Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science *287*, 2262–2267.

Hayashida, T., Nakahara, K., Mondal, M.S., Date, Y., Nakazato, M., Kojima, M., Kangawa, K., and Murakami, N. (2002). Ghrelin in neonatal rats: distribution in stomach and its possible role. The Journal of Endocrinology *173*, 239–245.

Hebb, D.O. (1949). The Organization of Behavior: A Neuropsychological Theory (John Wiley & Sons).

Heine, M., Groc, L., Frischknecht, R., Béïque, J.-C., Lounis, B., Rumbaugh, G., Huganir, R.L., Cognet, L., and Choquet, D. (2008). Surface mobility of postsynaptic AMPARs tunes synaptic transmission. Science *320*, 201–205.

Henley, J.M. (2003). Proteins interactions implicated in AMPA receptor trafficking: a clear destination and an improving route map. Neuroscience Research *45*, 243–254.

Henley, J.M., Barker, E. a, and Glebov, O.O. (2011). Routes, destinations and delays: recent advances in AMPA receptor trafficking. Trends in Neurosciences *34*, 258–268.

Hewson, A.K., and Dickson, S.L. (2000). Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. Journal of Neuroendocrinology *12*, 1047–1049.

Hillman, J.B., Tong, J., and Tschop, M. (2011). Ghrelin biology and its role in weight-related disorders. Discovery Medicine *11*, 521–528.

Hollmann, M., Hartley, M., and Heinemann, S. (1991). Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. Science *252*, 851–853.

Hollmann, M., and Heinemann, S. (1994). Cloned glutamate receptors. Annual Review of Neuroscience *17*, 31–108.

Hollmann, M., O'Shea-Greenfield, A., Rogers, S.W., and Heinemann, S. (1989). Cloning by functional expression of a member of the glutamate receptor family. Nature *342*, 643–648.

Holst, B., Cygankiewicz, A., Jensen, T.H., Ankersen, M., and Schwartz, T.W. (2003). High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. Molecular Endocrinology *17*, 2201–2210.

Holst, B., Egerod, K.L., Schild, E., Vickers, S.P., Cheetham, S., Gerlach, L.-O., Storjohann, L., Stidsen, C.E., Jones, R., Beck-Sickinger, A.G., et al. (2007). GPR39 signaling is stimulated by zinc ions but not by obestatin. Endocrinology *148*, 13–20.

Holst, B., Holliday, N.D., Bach, A., Elling, C.E., Cox, H.M., and Schwartz, T.W. (2004). Common structural basis for constitutive activity of the ghrelin receptor family. The Journal of Biological Chemistry *279*, 53806–53817.

Holst, B., Lang, M., Brandt, E., Bach, A., Howard, A., Frimurer, T.M., Beck-Sickinger, A., and Schwartz, T.W. (2006). Ghrelin receptor inverse agonists: identification of an active peptide core and its interaction epitopes on the receptor. Molecular Pharmacology *70*, 936–946.

Holst, B., and Schwartz, T.W. (2006). Ghrelin receptor mutations--too little height and too much hunger. The Journal of Clinical Investigation *116*, 637–641.

Hosoda, H., Kojima, M., Matsuo, H., and Kangawa, K. (2000a). Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochemical and Biophysical Research Communications *279*, 909–913.

Hosoda, H., Kojima, M., Matsuo, H., and Kangawa, K. (2000b). Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. The Journal of Biological Chemistry *275*, 21995–22000.

Hosoda, H., Kojima, M., Mizushima, T., Shimizu, S., and Kangawa, K. (2003). Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. The Journal of Biological Chemistry *278*, 64–70.

Howard, a D., Feighner, S.D., Cully, D.F., Arena, J.P., Liberator, P. a, Rosenblum, C.I., Hamelin, M., Hreniuk, D.L., Palyha, O.C., Anderson, J., et al. (1996). A receptor in pituitary and hypothalamus that functions in growth hormone release. Science *273*, 974–977.

Howard, A.D., McAllister, G., Feighner, S.D., Liu, Q., Nargund, R.P., Van der Ploeg, L.H., and Patchett, A.A. (2001). Orphan G-protein-coupled receptors and natural ligand discovery. Trends in Pharmacological Sciences *22*, 132–140.

Hsia, A.Y., Malenka, R.C., and Nicoll, R.A. (1998). Development of excitatory circuitry in the hippocampus. Journal of Neurophysiology *79*, 2013–2024.

Hu, Z., Cha, S.H., Van Haasteren, G., Wang, J., and Lane, M.D. (2005). Effect of centrally administered C75, a fatty acid synthase inhibitor, on ghrelin secretion and its downstream effects. Proceedings of the National Academy of Sciences of the United States of America *102*, 3972–3977.

Hudmon, A., Lebel, E., Roy, H., Sik, A., Schulman, H., Waxham, M.N., and De Koninck, P. (2005). A mechanism for Ca2+/calmodulin-dependent protein kinase II clustering at synaptic and nonsynaptic sites based on self-association. The Journal of Neuroscience *25*, 6971–6983.

Isaac, J.T., Crair, M.C., Nicoll, R.A., and Malenka, R.C. (1997). Silent synapses during development of thalamocortical inputs. Neuron *18*, 269–280.

Isidro, M.L., and Cordido, F. (2006). Growth hormone secretagogues. Combinatorial Chemistry & High Throughput Screening *9*, 175–180.

Jackson, A.C., and Nicoll, R.A. (2011). The expanding social network of ionotropic glutamate receptors: TARPs and other transmembrane auxiliary subunits. Neuron *70*, 178–199.

Jeffery, P.L., Duncan, R.P., Yeh, A.H., Jaskolski, R.A., Hammond, D.S., Herington, A.C., and Chopin, L.K. (2005a). Expression of the ghrelin axis in the mouse: an exon 4-deleted mouse proghrelin variant encodes a novel C terminal peptide. Endocrinology *146*, 432–440.

Jeffery, P.L., Murray, R.E., Yeh, A.H., McNamara, J.F., Duncan, R.P., Francis, G.D., Herington, A.C., and Chopin, L.K. (2005b). Expression and function of the ghrelin axis, including a novel preproghrelin isoform, in human breast cancer tissues and cell lines. Endocrine-related Cancer *12*, 839–850.

Jerlhag, E., Egecioglu, E., Dickson, S.L., Douhan, A., Svensson, L., and Engel, J.A. (2007). Ghrelin administration into tegmental areas stimulates locomotor activity and increases extracellular concentration of dopamine in the nucleus accumbens. Addiction Biology *12*, 6–16.

Jiang, H., Betancourt, L., and Smith, R.G. (2006). Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. Molecular Endocrinology *20*, 1772–1785.

Jiang, M., Deng, L., and Chen, G. (2004). High Ca(2+)-phosphate transfection efficiency enables single neuron gene analysis. Gene Therapy *11*, 1303–1311.

Jin, W., Ge, W.-P., Xu, J., Cao, M., Peng, L., Yung, W., Liao, D., Duan, S., Zhang, M., and Xia, J. (2006). Lipid binding regulates synaptic targeting of PICK1, AMPA receptor trafficking, and synaptic plasticity. The Journal of Neuroscience *26*, 2380–2390.

Kaji, H., Tai, S., Okimura, Y., Iguchi, G., Takahashi, Y., Abe, H., and Chihara, K. (1998). Cloning and characterization of the 5’-flanking region of the human growth hormone secretagogue receptor gene. The Journal of Biological Chemistry *273*, 33885–33888.

Kamegai, J., Tamura, H., Shimizu, T., Ishii, S., Sugihara, H., and Oikawa, S. (2004). Effects of insulin, leptin, and glucagon on ghrelin secretion from isolated perfused rat stomach. Regulatory Peptides *119*, 77–81.

Kanamoto, N., Akamizu, T., Tagami, T., Hataya, Y., Moriyama, K., Takaya, K., Hosoda, H., Kojima, M., Kangawa, K., and Nakao, K. (2004). Genomic structure and characterization of the 5’-flanking region of the human ghrelin gene. Endocrinology *145*, 4144–4153.

Kanehisa, M., Akiyoshi, J., Kitaichi, T., Matsushita, H., Tanaka, E., Kodama, K., Hanada, H., and Isogawa, K. (2006). Administration of antisense DNA for ghrelin causes an antidepressant and anxiolytic response in rats. Progress in Neuro-psychopharmacology & Biological Psychiatry *30*, 1403–1407.

Katayama, M., Nogami, H., Nishiyama, J., Kawase, T., and Kawamura, K. (2000). Developmentally and regionally regulated expression of growth hormone secretagogue receptor mRNA in rat brain and pituitary gland. Neuroendocrinology *72*, 333–340.

Katayama, T., Shimamoto, S., Oda, H., Nakahara, K., Kangawa, K., and Murakami, N. (2007). Glucagon receptor expression and glucagon stimulation of ghrelin secretion in rat stomach. Biochemical and Biophysical Research Communications *357*, 865–870.

Kauer, J.A., and Malenka, R.C. (2006). LTP: AMPA receptors trading places. Nature Neuroscience *9*, 593–594.

Kelleher, R.J., Govindarajan, A., and Tonegawa, S. (2004). Translational regulatory mechanisms in persistent forms of synaptic plasticity. Neuron *44*, 59–73.

Kennedy, M.J., Davison, I.G., Robinson, C.G., and Ehlers, M.D. (2010). Syntaxin-4 defines a domain for activity-dependent exocytosis in dendritic spines. Cell *141*, 524–535.

Kenny, P.J. (2011). Common cellular and molecular mechanisms in obesity and drug addiction. Nature Reviews Neuroscience *12*, 638–651.

Kern, A., Albarran-Zeckler, R., Walsh, H.E., and Smith, R.G. (2012). Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. Neuron *73*, 317–332.

Kessels, H.W., and Malinow, R. (2009). Synaptic AMPA receptor plasticity and behavior. Neuron *61*, 340–350.

Kim, C.H., Chung, H.J., Lee, H.K., and Huganir, R.L. (2001). Interaction of the AMPA receptor subunit GluR2/3 with PDZ domains regulates hippocampal long-term depression. Proceedings of the National Academy of Sciences of the United States of America *98*, 11725–11730.

Kim, E.-K., Miller, I., Aja, S., Landree, L.E., Pinn, M., McFadden, J., Kuhajda, F.P., Moran, T.H., and Ronnett, G. V (2004a). C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase. The Journal of Biological Chemistry *279*, 19970–19976.

Kim, H.-S., Jang, H.-J., Cho, K.-H., Hahn, S.J., Kim, M.-J., Yoon, S.H., Jo, Y.-H., Kim, M.-S., and Rhie, D.-J. (2006). Serotonin inhibits the induction of NMDA receptor-dependent long-term potentiation in the rat primary visual cortex. Brain Research *1103*, 49–55.

Kim, M.-S., Park, J.-Y., Namkoong, C., Jang, P.-G., Ryu, J.-W., Song, H.-S., Yun, J.-Y., Namgoong, I.-S., Ha, J., Park, I.-S., et al. (2004b). Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. Nature Medicine *10*, 727–733.

Kishimoto, M., Okimura, Y., Nakata, H., Kudo, T., Iguchi, G., Takahashi, Y., Kaji, H., and Chihara, K. (2003). Cloning and characterization of the 5(')-flanking region of the human ghrelin gene. Biochemical and Biophysical Research Communications *305*, 186–192.

Kohda, K., Wang, Y., and Yuzaki, M. (2000). Mutation of a glutamate receptor motif reveals its role in gating and delta2 receptor channel properties. Nature Neuroscience *3*, 315–322.

Kohno, D., Gao, H.-Z., Muroya, S., Kikuyama, S., and Yada, T. (2003). Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca2+ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. Diabetes *52*, 948–956.

Kojima, M. (2008). The discovery of ghrelin--a personal memory. Regulatory Peptides *145*, 2–6.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature *402*, 656–660.

Kojima, M., Hosoda, H., and Kangawa, K. (2001). Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. Hormone Research *56 Suppl 1*, 93–97.

Kojima, M., and Kangawa, K. (2005). Ghrelin: structure and function. Physiological Reviews *85*, 495–522.

Kojima, M., and Kangawa, K. (2010). Ghrelin: more than endogenous growth hormone secretagogue. Annals of the New York Academy of Sciences *1200*, 140–148.

Kojima, T., Matsumoto, M., Togashi, H., Tachibana, K., Kemmotsu, O., and Yoshioka, M. (2003). Fluvoxamine suppresses the long-term potentiation in the hippocampal CA1 field of anesthetized rats: an effect mediated via 5-HT1A receptors. Brain Research *959*, 165–168.

Korbonits, M., Bustin, S.A., Kojima, M., Jordan, S., Adams, E.F., Lowe, D.G., Kangawa, K., and Grossman, A.B. (2001a). The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. The Journal of Clinical Endocrinology and Metabolism *86*, 881–887.

Korbonits, M., Ciccarelli, E., Ghigo, E., and Grossman, A.B. (1999). The growth hormone secretagogue receptor. Growth Hormone & IGF Research *9 Suppl A*, 93–99.

Korbonits, M., Goldstone, A.P., Gueorguiev, M., and Grossman, A.B. (2004). Ghrelin-a hormone with multiple functions. Frontiers in Neuroendocrinology *25*, 27–68.

Korbonits, M., Kojima, M., Kangawa, K., and Grossman, A.B. (2001b). Presence of ghrelin in normal and adenomatous human pituitary. Endocrine *14*, 101–104.

Kristensen, A.S., Jenkins, M.A., Banke, T.G., Schousboe, A., Makino, Y., Johnson, R.C., Huganir, R., and Traynelis, S.F. (2011). Mechanism of Ca2+/calmodulin-dependent kinase II regulation of AMPA receptor gating. Nature Neuroscience *14*, 727–735.

Kristenssson, E., Sundqvist, M., Astin, M., Kjerling, M., Mattsson, H., Dornonville de la Cour, C., Håkanson, R., and Lindström, E. (2006). Acute psychological stress raises plasma ghrelin in the rat. Regulatory Peptides *134*, 114–117.

Krsek, M., Rosická, M., Papezová, H., Krízová, J., Kotrlíková, E., Haluz'k, M., Justová, V., Lacinová, Z., and Jarkovská, Z. (2003). Plasma ghrelin levels and malnutrition: a comparison of two etiologies. Eating and Weight Disorders *8*, 207–211.

Lattuada, D., Crotta, K., Tonna, N., Casnici, C., Benfante, R., Fornasari, D., Bianco, F., Longhi, R., and Marelli, O. (2013). The Expression of GHS-R in Primary Neurons Is Dependent upon Maturation Stage and Regional Localization. PloS One *8*, e64183.

Lee, H., Te, C., Koshy, S., Teixeira, J.A., Pi-Sunyer, F.X., and Laferrère, B. (2006a). Does ghrelin really matter after bariatric surgery? Surgery for Obesity and Related Diseases *2*, 538–548.

Lee, H.-J., and Zheng, J.J. (2010). PDZ domains and their binding partners: structure, specificity, and modification. Cell Communication and Signaling *8*, 8.

Lee, H.K., Barbarosie, M., Kameyama, K., Bear, M.F., and Huganir, R.L. (2000). Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. Nature *405*, 955–959.

Lee, H.-K., Takamiya, K., Han, J.-S., Man, H., Kim, C.-H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R.S., et al. (2003). Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. Cell *112*, 631–643.

Lee, H.-K., Takamiya, K., Kameyama, K., He, K., Yu, S., Rossetti, L., Wilen, D., and Huganir, R.L. (2007). Identification and characterization of a novel phosphorylation site on the GluR1 subunit of AMPA receptors. Molecular and Cellular Neurosciences *36*, 86–94.

Lee, S.-J.R., Escobedo-Lozoya, Y., Szatmari, E.M., and Yasuda, R. (2009). Activation of CaMKII in single dendritic spines during long-term potentiation. Nature *458*, 299–304.

Lee, Y.S., Challis, B.G., Thompson, D.A., Yeo, G.S.H., Keogh, J.M., Madonna, M.E., Wraight, V., Sims, M., Vatin, V., Meyre, D., et al. (2006b). A POMC variant implicates beta-melanocyte-stimulating hormone in the control of human energy balance. Cell Metabolism *3*, 135–140.

van der Lely, A.J., Tschöp, M., Heiman, M.L., and Ghigo, E. (2004). Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocrine Reviews *25*, 426–457.

Leonetti, F., Silecchia, G., Iacobellis, G., Ribaudo, M.C., Zappaterreno, A., Tiberti, C., Iannucci, C.V., Perrotta, N., Bacci, V., Basso, M.S., et al. (2003). Different plasma ghrelin levels after laparoscopic gastric bypass and adjustable gastric banding in morbid obese subjects. The Journal of Clinical Endocrinology and Metabolism *88*, 4227–4231.

Lerma, J. (2006). Kainate receptor physiology. Current Opinion in Pharmacology *6*, 89–97.

Leung, P.-K., Chow, K.B.S., Lau, P.-N., Chu, K.-M., Chan, C.-B., Cheng, C.H.K., and Wise, H. (2007). The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor. Cellular Signalling *19*, 1011–1022.

Lin, D.-T., and Huganir, R.L. (2007). PICK1 and phosphorylation of the glutamate receptor 2 (GluR2) AMPA receptor subunit regulates GluR2 recycling after NMDA receptor-induced internalization. The Journal of Neuroscience *27*, 13903–13908.

Lin, D.-T., Makino, Y., Sharma, K., Hayashi, T., Neve, R., Takamiya, K., and Huganir, R.L. (2009). Regulation of AMPA receptor extrasynaptic insertion by 4.1N, phosphorylation and palmitoylation. Nature Neuroscience *12*, 879–887.

Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. Nature Reviews Neuroscience *3*, 175–190.

Lissin, D. V, Gomperts, S.N., Carroll, R.C., Christine, C.W., Kalman, D., Kitamura, M., Hardy, S., Nicoll, R.A., Malenka, R.C., and Von Zastrow, M. (1998). Activity differentially regulates the surface expression of synaptic AMPA and NMDA glutamate receptors. Proceedings of the National Academy of Sciences of the United States of America *95*, 7097–7102.

Liu, T., Kong, D., Shah, B.P., Ye, C., Koda, S., Saunders, A., Ding, J.B., Yang, Z., Sabatini, B.L., and Lowell, B.B. (2012). Fasting activation of AgRP neurons requires NMDA receptors and involves spinogenesis and increased excitatory tone. Neuron *73*, 511–522.

Locke, W., Kirgis, H.D., Bowers, C.Y., and Abdoh, A.A. (1995). Intracerebroventricular growth-hormone-releasing peptide-6 stimulates eating without affecting plasma growth hormone responses in rats. Life Sciences *56*, 1347–1352.

Lomenick, J.P., Melguizo, M.S., Mitchell, S.L., Summar, M.L., and Anderson, J.W. (2009). Effects of meals high in carbohydrate, protein, and fat on ghrelin and peptide YY secretion in prepubertal children. The Journal of Clinical Endocrinology and Metabolism *94*, 4463–4471.

Lu, S., Guan, J.-L., Wang, Q.-P., Uehara, K., Yamada, S., Goto, N., Date, Y., Nakazato, M., Kojima, M., Kangawa, K., et al. (2002). Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. Neuroscience Letters *321*, 157–160.

Lu, W., Isozaki, K., Roche, K.W., and Nicoll, R.A. (2010). Synaptic targeting of AMPA receptors is regulated by a CaMKII site in the first intracellular loop of GluA1. Proceedings of the National Academy of Sciences of the United States of America *107*, 22266–22271.

Lu, W., Man, H., Ju, W., Trimble, W.S., MacDonald, J.F., and Wang, Y.T. (2001). Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. Neuron *29*, 243–254.

Lu, W., and Roche, K.W. (2012). Posttranslational regulation of AMPA receptor trafficking and function. Current Opinion in Neurobiology *22*, 470–479.

Lüscher, C., and Malenka, R.C. (2012). NMDA receptor-dependent long-term potentiation and long-term depression (LTP/LTD). Cold Spring Harbor Perspectives in Biology *4*,.

Lutter, M., Sakata, I., Osborne-Lawrence, S., Rovinsky, S.A., Anderson, J.G., Jung, S., Birnbaum, S., Yanagisawa, M., Elmquist, J.K., Nestler, E.J., et al. (2008). The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nature Neuroscience *11*, 752–753.

Lynch, M.A. (2004). Long-term potentiation and memory. Physiological Reviews *84*, 87–136.

Mack, V., Burnashev, N., Kaiser, K.M., Rozov, A., Jensen, V., Hvalby, O., Seeburg, P.H., Sakmann, B., and Sprengel, R. (2001). Conditional restoration of hippocampal synaptic potentiation in Glur-A-deficient mice. Science *292*, 2501–2504.

Makino, H., and Malinow, R. (2009). AMPA receptor incorporation into synapses during LTP: the role of lateral movement and exocytosis. Neuron *64*, 381–390.

Malenka, R.C. (2003). Synaptic plasticity and AMPA receptor trafficking. Annals of the New York Academy of Sciences *1003*, 1–11.

Malenka, R.C., and Bear, M.F. (2004). LTP and LTD: an embarrassment of riches. Neuron *44*, 5–21.

Maletic-Savatic, M., Koothan, T., and Malinow, R. (1998). Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part II: mediation by calcium/calmodulin-dependent protein kinase II. The Journal of Neuroscience *18*, 6814–6821.

Malik, S., McGlone, F., Bedrossian, D., and Dagher, A. (2008). Ghrelin modulates brain activity in areas that control appetitive behavior. Cell Metabolism *7*, 400–409.

Malinow, R., Hayashi, Y., Maletic-Savatic, M., Zaman, S.H., Poncer, J.-C., Shi, S.-H., Esteban, J.A., Osten, P., and Seidenman, K. (2010). Introduction of green fluorescent protein (GFP) into hippocampal neurons through viral infection. Cold Spring Harbor Protocols *2010*, pdb.prot5406.

Malinow, R., and Malenka, R.C. (2002). AMPA receptor trafficking and synaptic plasticity. Annual Review of Neuroscience *25*, 103–126.

Mammen, a L., Kameyama, K., Roche, K.W., and Huganir, R.L. (1997). Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. The Journal of Biological Chemistry *272*, 32528–32533.

Man, H.-Y., Wang, Q., Lu, W.-Y., Ju, W., Ahmadian, G., Liu, L., D'Souza, S., Wong, T.P., Taghibiglou, C., Lu, J., et al. (2003). Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. Neuron *38*, 611–624.

Mansour, M., Nagarajan, N., Nehring, R.B., Clements, J.D., and Rosenmund, C. (2001). Heteromeric AMPA receptors assemble with a preferred subunit stoichiometry and spatial arrangement. Neuron *32*, 841–853.

Martin, S.J., Grimwood, P.D., and Morris, R.G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. Annual Review of Neuroscience *23*, 649–711.

Marzullo, P., Verti, B., Savia, G., Walker, G.E., Guzzaloni, G., Tagliaferri, M., Di Blasio, A., and Liuzzi, A. (2004). The relationship between active ghrelin levels and human obesity involves alterations in resting energy expenditure. The Journal of Clinical Endocrinology and Metabolism *89*, 936–939.

Matsuda, S., Launey, T., Mikawa, S., and Hirai, H. (2000). Disruption of AMPA receptor GluR2 clusters following long-term depression induction in cerebellar Purkinje neurons. The EMBO Journal *19*, 2765–2774.

Matsuda, S., Mikawa, S., and Hirai, H. (1999). Phosphorylation of serine-880 in GluR2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein. Journal of Neurochemistry *73*, 1765–1768.

Matthews, R.P., Guthrie, C.R., Wailes, L.M., Zhao, X., Means, A.R., and McKnight, G.S. (1994). Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Molecular and Cellular Biology *14*, 6107–6116.

Mayer, M.L. (2006). Glutamate receptors at atomic resolution. Nature *440*, 456–462.

Mazzocchi, G., Neri, G., Rucinski, M., Rebuffat, P., Spinazzi, R., Malendowicz, L.K., and Nussdorfer, G.G. (2004). Ghrelin enhances the growth of cultured human adrenal zona glomerulosa cells by exerting MAPK-mediated proliferogenic and antiapoptotic effects. Peptides *25*, 1269–1277.

McDonald, B.J., Chung, H.J., and Huganir, R.L. (2001). Identification of protein kinase C phosphorylation sites within the AMPA receptor GluR2 subunit. Neuropharmacology *41*, 672–679.

McKee, K.K., Palyha, O.C., Feighner, S.D., Hreniuk, D.L., Tan, C.P., Phillips, M.S., Smith, R.G., Van der Ploeg, L.H., and Howard, A.D. (1997a). Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. Molecular Endocrinology *11*, 415–423.

McKee, K.K., Tan, C.P., Palyha, O.C., Liu, J., Feighner, S.D., Hreniuk, D.L., Smith, R.G., Howard, A.D., and Van der Ploeg, L.H. (1997b). Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. Genomics *46*, 426–434.

Mear, Y., Enjalbert, A., and Thirion, S. (2013). GHS-R1a constitutive activity and its physiological relevance. Frontiers in Neuroscience *7*, 1–7.

Meng, Y., Zhang, Y., and Jia, Z. (2003). Synaptic transmission and plasticity in the absence of AMPA glutamate receptor GluR2 and GluR3. Neuron *39*, 163–176.

Merrill, M.A., Chen, Y., Strack, S., and Hell, J.W. (2005). Activity-driven postsynaptic translocation of CaMKII. Trends in Pharmacological Sciences *26*, 645–653.

Moran, T.H., and Gao, S. (2006). Looking for food in all the right places? Cell Metabolism *3*, 233–234.

Mori, K., Togashi, H., Kojima, T., Matsumoto, M., Ohashi, S., Ueno, K., and Yoshioka, M. Different effects of anxiolytic agents, diazepam and 5-HT(1A) agonist tandospirone, on hippocampal long-term potentiation in vivo. Pharmacology, Biochemistry, and Behavior *69*, 367–372.

Mori, K., Yoshimoto, A., Takaya, K., Hosoda, K., Ariyasu, H., Yahata, K., Mukoyama, M., Sugawara, A., Hosoda, H., Kojima, M., et al. (2000). Kidney produces a novel acylated peptide, ghrelin. FEBS Letters *486*, 213–216.

Moult, P.R., Cross, A., Santos, S.D., Carvalho, A.-L., Lindsay, Y., Connolly, C.N., Irving, A.J., Leslie, N.R., and Harvey, J. (2010). Leptin regulates AMPA receptor trafficking via PTEN inhibition. The Journal of Neuroscience *30*, 4088–4101.

Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B., and Cone, R.D. (1994). Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Molecular Endocrinology *8*, 1298–1308.

Muccioli, G., Baragli, A., Granata, R., Papotti, M., and Ghigo, E. (2007). Heterogeneity of ghrelin/growth hormone secretagogue receptors. Toward the understanding of the molecular identity of novel ghrelin/GHS receptors. Neuroendocrinology *86*, 147–164.

Muccioli, G., Broglio, F., Valetto, M.R., Ghè, C., Catapano, F., Graziani, A., Papotti, M., Bisi, G., Deghenghi, R., and Ghigo, E. (2000). Growth hormone-releasing peptides and the cardiovascular system. Annales D'endocrinologie *61*, 27–31.

Muccioli, G., Ghè, C., Ghigo, M.C., Papotti, M., Arvat, E., Boghen, M.F., Nilsson, M.H., Deghenghi, R., Ong, H., and Ghigo, E. (1998). Specific receptors for synthetic GH secretagogues in the human brain and pituitary gland. The Journal of Endocrinology *157*, 99–106.

Müller, E.E., Locatelli, V., and Cocchi, D. (1999). Neuroendocrine control of growth hormone secretion. Physiological Reviews *79*, 511–607.

Müller, T.D., Müller, A., Yi, C.-X., M Habegger, K., Meyer, C.W., Gaylinn, B.D., Finan, B., Heppner, K., Trivedi, C., Bielohuby, M., et al. (2013). The orphan receptor Gpr83 regulates systemic energy metabolism via ghrelin-dependent and ghrelin-independent mechanisms. Nature Communications *4*, 1968.

Nakai, N., Kaneko, M., Nakao, N., Fujikawa, T., Nakashima, K., Ogata, M., and Tanaka, M. (2004). Identification of promoter region of ghrelin gene in human medullary thyroid carcinoma cell line. Life Sciences *75*, 2193–2201.

Nakanishi, S., and Masu, M. (1994). Molecular diversity and functions of glutamate receptors. Annual Review of Biophysics and Biomolecular Structure *23*, 319–348.

Nakashima, K., Akiyoshi, J., Hatano, K., Hanada, H., Tanaka, Y., Tsuru, J., Matsushita, H., Kodama, K., and Isogawa, K. (2008). Ghrelin gene polymorphism is associated with depression, but not panic disorder. Psychiatric Genetics *18*, 257.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. Nature *409*, 194–198.

Naleid, A.M., Grace, M.K., Cummings, D.E., and Levine, A.S. (2005). Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. Peptides *26*, 2274–2279.

Neary, N.M., Goldstone, A.P., and Bloom, S.R. (2004). Appetite regulation: from the gut to the hypothalamus. Clinical Endocrinology *60*, 153–160.

Nemeth, E.F. (2006). The parathyroid polyhormone hypothesis revisited. Kidney International. Supplement S22–8.

Nicoll, R.A., Tomita, S., and Bredt, D.S. (2006). Auxiliary subunits assist AMPA-type glutamate receptors. Science *311*, 1253–1256.

Nicoll, R.A., and Roche, K.W. (2013). Long-term potentiation: peeling the onion. Neuropharmacology.

Niswender, C.M., and Conn, P.J. (2010). Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annual Review of Pharmacology and Toxicology *50*, 295–322.

Nusser, Z., Lujan, R., Laube, G., Roberts, J.D., Molnar, E., and Somogyi, P. (1998). Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. Neuron *21*, 545–559.

Oh, M.C., Derkach, V. a, Guire, E.S., and Soderling, T.R. (2006). Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for long-term potentiation. The Journal of Biological Chemistry *281*, 752–758.

Oh, M.C., and Derkach, V.A. (2005). Dominant role of the GluR2 subunit in regulation of AMPA receptors by CaMKII. Nature Neuroscience *8*, 853–854.

Ohno, T., Mochiki, E., and Kuwano, H. (2010). The roles of motilin and ghrelin in gastrointestinal motility. International Journal of Peptides *2010*,.

Okada, K., Ishii, S., Minami, S., Sugihara, H., Shibasaki, T., and Wakabayashi, I. (1996). Intracerebroventricular administration of the growth hormone-releasing peptide KP-102 increases food intake in free-feeding rats. Endocrinology *137*, 5155–5158.

Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., and Barsh, G.S. (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science *278*, 135–138.

Olson, G.A., Olson, R.D., Kastin, A.J., and Coy, D.H. (1979). Endogenous opiates: through 1978. Neuroscience and Biobehavioral Reviews *3*, 285–299.

Olszewski, P.K., Schiöth, H.B., and Levine, A.S. (2008). Ghrelin in the CNS: from hunger to a rewarding and memorable meal? Brain Research Reviews *58*, 160–170.

Ong, H., Bodart, V., McNicoll, N., Lamontagne, D., and Bouchard, J.F. (1998a). Binding sites for growth hormone-releasing peptide. Growth Hormone & IGF Research *8 Suppl B*, 137–140.

Ong, H., McNicoll, N., Escher, E., Collu, R., Deghenghi, R., Locatelli, V., Ghigo, E., Muccioli, G., Boghen, M., and Nilsson, M. (1998b). Identification of a pituitary growth hormone-releasing peptide (GHRP) receptor subtype by photoaffinity labeling. Endocrinology *139*, 432–435.

Opazo, P., Labrecque, S., Tigaret, C.M., Frouin, A., Wiseman, P.W., De Koninck, P., and Choquet, D. (2010). CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. Neuron *67*, 239–252.

Opazo, P., Sainlos, M., and Choquet, D. (2012). Regulation of AMPA receptor surface diffusion by PSD-95 slots. Current Opinion in Neurobiology *22*, 453–460.

Opazo, P., Watabe, A.M., Grant, S.G.N., and O'Dell, T.J. (2003). Phosphatidylinositol 3-kinase regulates the induction of long-term potentiation through extracellular signal-related kinase-independent mechanisms. The Journal of Neuroscience *23*, 3679–3688.

Otmakhov, N., Tao-Cheng, J.-H., Carpenter, S., Asrican, B., Dosemeci, A., Reese, T.S., and Lisman, J. (2004). Persistent accumulation of calcium/calmodulin-dependent protein kinase II in dendritic spines after induction of NMDA receptor-dependent chemical long-term potentiation. The Journal of Neuroscience *24*, 9324–9331.

Otto, B., Cuntz, U., Fruehauf, E., Wawarta, R., Folwaczny, C., Riepl, R.L., Heiman, M.L., Lehnert, P., Fichter, M., and Tschöp, M. (2001). Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. European Journal of Endocrinology *145*, 669–673.

Ozawa, A., Cai, Y., and Lindberg, I. (2007). Production of bioactive peptides in an in vitro system. Analytical Biochemistry *366*, 182–189.

Palyha, O.C., Feighner, S.D., Tan, C.P., McKee, K.K., Hreniuk, D.L., Gao, Y.D., Schleim, K.D., Yang, L., Morriello, G.J., Nargund, R., et al. (2000). Ligand activation domain of human orphan growth hormone (GH) secretagogue receptor (GHS-R) conserved from Pufferfish to humans. Molecular Endocrinology *14*, 160–169.

Pan, W., Tu, H., and Kastin, A.J. (2006). Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin. Peptides *27*, 911–916.

Pantel, J., Legendre, M., Cabrol, S., Hilal, L., Hajaji, Y., Morisset, S., Nivot, S., Vie-Luton, M.-P., Grouselle, D., De Kerdanet, M., et al. (2006). Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. The Journal of Clinical Investigation *116*, 760–768.

Papotti, M., Ghè, C., Cassoni, P., Catapano, F., Deghenghi, R., Ghigo, E., and Muccioli, G. (2000). Growth hormone secretagogue binding sites in peripheral human tissues. The Journal of Clinical Endocrinology and Metabolism *85*, 3803–3807.

Park, M., Penick, E.C., Edwards, J.G., Kauer, J.A., and Ehlers, M.D. (2004). Recycling endosomes supply AMPA receptors for LTP. Science *305*, 1972–1975.

Passafaro, M., Piëch, V., and Sheng, M. (2001). Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. Nature Neuroscience *4*, 917–926.

Pastalkova, E., Serrano, P., Pinkhasova, D., Wallace, E., Fenton, A.A., and Sacktor, T.C. (2006). Storage of spatial information by the maintenance mechanism of LTP. Science *313*, 1141–1144.

Patchett, A.A., Nargund, R.P., Tata, J.R., Chen, M.H., Barakat, K.J., Johnston, D.B., Cheng, K., Chan, W.W., Butler, B., and Hickey, G. (1995). Design and biological activities of L-163,191 (MK-0677): a potent, orally active growth hormone secretagogue. Proceedings of the National Academy of Sciences of the United States of America *92*, 7001–7005.

Patterson, M., Bloom, S.R., and Gardiner, J. V (2011). Ghrelin and appetite control in humans--potential application in the treatment of obesity. Peptides *32*, 2290–2294.

Pavlides, C., Westlind-Danielsson, A.I., Nyborg, H., and McEwen, B.S. (1991). Neonatal hyperthyroidism disrupts hippocampal LTP and spatial learning. Experimental Brain Research. Experimentelle Hirnforschung. Expérimentation Cérébrale *85*, 559–564.

Peça, J., Feliciano, C., Ting, J.T., Wang, W., Wells, M.F., Venkatraman, T.N., Lascola, C.D., Fu, Z., and Feng, G. (2011). Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. Nature *472*, 437–442.

Peino, R., Baldelli, R., Rodriguez-Garcia, J., Rodriguez-Segade, S., Kojima, M., Kangawa, K., Arvat, E., Ghigo, E., Dieguez, C., and Casanueva, F.F. (2000). Ghrelin-induced growth hormone secretion in humans. European Journal of Endocrinology *143*, R11–4.

Pemberton, C., Wimalasena, P., Yandle, T., Soule, S., and Richards, M. (2003). C-terminal pro-ghrelin peptides are present in the human circulation. Biochemical and Biophysical Research Communications *310*, 567–573.

Perello, M., Sakata, I., Birnbaum, S., Chuang, J.-C., Osborne-Lawrence, S., Rovinsky, S.A., Woloszyn, J., Yanagisawa, M., Lutter, M., and Zigman, J.M. (2010). Ghrelin increases the rewarding value of high-fat diet in an orexin-dependent manner. Biological Psychiatry *67*, 880–886.

Perelló, M., and Zigman, J.M. (2012). The role of ghrelin in reward-based eating. Biological Psychiatry *72*, 347–353.

Perez, J.L., Khatri, L., Chang, C., Srivastava, S., Osten, P., and Ziff, E.B. (2001). PICK1 targets activated protein kinase Calpha to AMPA receptor clusters in spines of hippocampal neurons and reduces surface levels of the AMPA-type glutamate receptor subunit 2. The Journal of Neuroscience□*21*, 5417–5428.

Petersen, P.S., Woldbye, D.P.D., Madsen, A.N., Egerod, K.L., Jin, C., Lang, M., Rasmussen, M., Beck-Sickinger, A.G., and Holst, B. (2009). In vivo characterization of high Basal signaling from the ghrelin receptor. Endocrinology *150*, 4920–4930.

Petersenn, S. (2002). Structure and regulation of the growth hormone secretagogue receptor. Minerva Endocrinologica *27*, 243–256.

Petralia, R.S., Esteban, J.A., Wang, Y.X., Partridge, J.G., Zhao, H.M., Wenthold, R.J., and Malinow, R. (1999). Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses. Nature Neuroscience *2*, 31–36.

Petrini, E.M., Lu, J., Cognet, L., Lounis, B., Ehlers, M.D., and Choquet, D. (2009). Endocytic trafficking and recycling maintain a pool of mobile surface AMPA receptors required for synaptic potentiation. Neuron *63*, 92–105.

Pettit, D.L., Perlman, S., and Malinow, R. (1994). Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. Science *266*, 1881–1885.

Pimenta, D.C., and Lebrun, I. (2007). Cryptides: buried secrets in proteins. Peptides *28*, 2403–2410.

Poncer, J., Esteban, J., and Malinow, R. (2002). Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by alpha-Ca2+/ calmodulin-dependent protein kinase II. Journal of Neuroscience *22*, 4406–4411.

Pong, S.S., Chaung, L.Y., Dean, D.C., Nargund, R.P., Patchett, A.A., and Smith, R.G. (1996). Identification of a new G-protein-linked receptor for growth hormone secretagogues. Molecular Endocrinology *10*, 57–61.

Ponimaskin, E.G., Profirovic, J., Vaiskunaite, R., Richter, D.W., and Voyno-yasenetskaya, T.A. (2002). 5-Hydroxytryptamine 4(a) Receptor Is Coupled to the Gα Subunit of Heterotrimeric G13 Protein*. The Journal of Biological Chemistry *277*, 20812-20819.

Popovic, V., Micic, D., Damjanovic, S., Djurovic, M., Simic, M., Gligorovic, M., Dieguez, C., and Casanueva, F.F. (1996). Evaluation of pituitary GH reserve with GHRP-6. Journal of Pediatric Endocrinology & Metabolism□*9 Suppl 3*, 289–298.

Portelli, J., Thielemans, L., Ver Donck, L., Loyens, E., Coppens, J., Aourz, N., Aerssens, J., Vermoesen, K., Clinckers, R., Schallier, A., et al. (2012). Inactivation of the constitutively active ghrelin receptor attenuates limbic seizure activity in rodents. Neurotherapeutics *9*, 658–672.

Priel, A., Kolleker, A., Ayalon, G., Gillor, M., Osten, P., and Stern-Bach, Y. (2005). Stargazin reduces desensitization and slows deactivation of the AMPA-type glutamate receptors. The Journal of Neuroscience *25*, 2682–2686.

Raffin-Sanson, M.L., De Keyzer, Y., and Bertagna, X. (2003). Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. European Journal of Endocrinology *149*, 79–90.

Rediger, A., Piechowski, C.L., Yi, C.-X., Tarnow, P., Strotmann, R., Grüters, A., Krude, H., Schöneberg, T., Tschöp, M.H., Kleinau, G., et al. (2011). Mutually opposite signal modulation by hypothalamic heterodimerization of ghrelin and melanocortin-3 receptors. The Journal of Biological Chemistry *286*, 39623–39631.

Rediger, A., Tarnow, P., Bickenbach, A., Schaefer, M., Krude, H., Gruters, A., and Biebermann, H. (2009). Heterodimerization of hypothalamic G-protein-coupled receptors involved in weight regulation. Obesity Facts *2*, 80–86.

del Rincon, J.P., Thorner, M.O., and Gaylinn, B.G. (2001). Motilin-related peptide and ghrelin: lessons from molecular techniques, peptide chemistry, and receptor biology. Gastroenterology *120*, 587–8.

Rioult-Pedotti, M.S., Friedman, D., and Donoghue, J.P. (2000). Learning-induced LTP in neocortex. Science *290*, 533–536.

Roche, K.W., O'Brien, R.J., Mammen, a L., Bernhardt, J., and Huganir, R.L. (1996). Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. Neuron *16*, 1179–1188.

Rodríguez-Pacheco, F., Luque, R.M., García-Navarro, S., Gracia-Navarro, F., Castaño, J.P., and Malagón, M.M. (2005). Ghrelin induces growth hormone (GH) secretion via nitric oxide (NO)/cGMP signaling. Annals of the New York Academy of Sciences *1040*, 452–453.

Rolland, Y., Abellan van Kan, G., Gillette-Guyonnet, S., and Vellas, B. (2011). Cachexia versus sarcopenia. Current Opinion in Clinical Nutrition and Metabolic Care *14*, 15–21.

Rongo, C., and Kaplan, J.M. (1999). CaMKII regulates the density of central glutamatergic synapses in vivo. Nature *402*, 195–199.

Rosenmund, C., Stern-Bach, Y., and Stevens, C.F. (1998). The tetrameric structure of a glutamate receptor channel. Science *280*, 1596–1599.

Rosická, M., Krsek, M., Matoulek, M., Jarkovská, Z., Marek, J., Justová, V., and Lacinová, Z. (2003). Serum ghrelin levels in obese patients: the relationship to serum leptin levels and soluble leptin receptors levels. Physiological Research / Academia Scientiarum Bohemoslovaca *52*, 61–66.

le Roux, C.W., Neary, N.M., Halsey, T.J., Small, C.J., Martinez-Isla, A.M., Ghatei, M.A., Theodorou, N.A., and Bloom, S.R. (2005). Ghrelin does not stimulate food intake in patients with surgical procedures involving vagotomy. The Journal of Clinical Endocrinology and Metabolism *90*, 4521–4524.

Rudolph, J., Esler, W.P., O'connor, S., Coish, P.D.G., Wickens, P.L., Brands, M., Bierer, D.E., Bloomquist, B.T., Bondar, G., Chen, L., et al. (2007). Quinazolinone derivatives as orally available ghrelin receptor antagonists for the treatment of diabetes and obesity. Journal of Medicinal Chemistry *50*, 5202–5216.

Rüter, J., Kobelt, P., Tebbe, J.J., Avsar, Y., Veh, R., Wang, L., Klapp, B.F., Wiedenmann, B., Taché, Y., and Mönnikes, H. (2003). Intraperitoneal injection of ghrelin induces Fos expression in the paraventricular nucleus of the hypothalamus in rats. Brain Research *991*, 26–33.

Sahu, A., Kalra, S.P., Crowley, W.R., and Kalra, P.S. (1988). Evidence that NPY-containing neurons in the brainstem project into selected hypothalamic nuclei: implication in feeding behavior. Brain Research *457*, 376–378.

Saito, E.-S., Kaiya, H., Tachibana, T., Tomonaga, S., Denbow, D.M., Kangawa, K., and Furuse, M. (2005). Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regulatory Peptides *125*, 201–208.

Sakata, I., Nakamura, K., Yamazaki, M., Matsubara, M., Hayashi, Y., Kangawa, K., and Sakai, T. (2002). Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. Peptides *23*, 531–536.

Sakata, I., Yamazaki, M., Inoue, K., Hayashi, Y., Kangawa, K., and Sakai, T. (2003). Growth hormone secretagogue receptor expression in the cells of the stomach-projected afferent nerve in the rat nodose ganglion. Neuroscience Letters *342*, 183–186.

Sands, W.A., and Palmer, T.M. (2008). Regulating gene transcription in response to cyclic AMP elevation. Cellular Signalling *20*, 460–466.

Sanhueza, M., McIntyre, C.C., and Lisman, J.E. (2007). Reversal of synaptic memory by Ca2+/calmodulin-dependent protein kinase II inhibitor. The Journal of Neuroscience *27*, 5190–5199.

Sanna, P.P., Cammalleri, M., Berton, F., Simpson, C., Lutjens, R., Bloom, F.E., and Francesconi, W. (2002). Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. The Journal of Neuroscience : the Official Journal of the Society for Neuroscience *22*, 3359–3365.

Santos, S.D., Carvalho, A.L., Caldeira, M. V, and Duarte, C.B. (2009). Regulation of AMPA receptors and synaptic plasticity. Neuroscience *158*, 105–125.

Sato, T., Nakamura, Y., Shiimura, Y., Ohgusu, H., Kangawa, K., and Kojima, M. (2012). Structure, regulation and function of ghrelin. Journal of Biochemistry *151*, 119–128.

Satou, M., Nakamura, Y., Ando, H., and Sugimoto, H. (2011). Understanding the functional significance of ghrelin processing and degradation. Peptides *32*, 2183–2190.

Schellekens, H., Finger, B.C., Dinan, T.G., and Cryan, J.F. (2012). Ghrelin signalling and obesity: at the interface of stress, mood and food reward. Pharmacology & Therapeutics *135*, 316–326.

Schellekens, H., Van Oeffelen, W.E.P.A., Dinan, T.G., and Cryan, J.F. (2013). Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signaling. The Journal of Biological Chemistry *288*, 181–191.

Schmid, S.M., Kott, S., Sager, C., Huelsken, T., and Hollmann, M. (2009). The glutamate receptor subunit delta2 is capable of gating its intrinsic ion channel as revealed by ligand binding domain transplantation. Proceedings of the National Academy of Sciences of the United States of America *106*, 10320–10325.

Schwartz, M.W., Woods, S.C., Porte, D., Seeley, R.J., and Baskin, D.G. (2000). Central nervous system control of food intake. Nature *404*, 661–671.

Seeburg, P.H. (1993). The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. Trends in Neurosciences *16*, 359–365.

Seidenman, K.J., Steinberg, J.P., Huganir, R., and Malinow, R. (2003). Glutamate receptor subunit 2 Serine 880 phosphorylation modulates synaptic transmission and mediates plasticity in CA1 pyramidal cells. The Journal of Neuroscience *23*, 9220–9228.

Seim, I., Herington, A.C., and Chopin, L.K. (2009). New insights into the molecular complexity of the ghrelin gene locus. Cytokine & Growth Factor Reviews *20*, 297–304.

Seino, S., Sakurai, H., Seino, Y., Tsuda, K., Tanigawa, K., Kuzuya, H., Goto, Y., and Imura, H. (1980). Starvation-induced changes of somatostatin, glucagon, and insulin secretion from the isolated perfused rat pancreas. Diabetes *29*, 323–325.

Serulle, Y., Zhang, S., Ninan, I., Puzzo, D., McCarthy, M., Khatri, L., Arancio, O., and Ziff, E.B. (2007). A GluR1-cGKII interaction regulates AMPA receptor trafficking. Neuron *56*, 670–688.

Sethumadhavan, K., Veeraragavan, K., and Bowers, C.Y. (1991). Demonstration and characterization of the specific binding of growth hormone-releasing peptide to rat anterior pituitary and hypothalamic membranes. Biochemical and Biophysical Research Communications *178*, 31–37.

Shearman, L.P., Wang, S.-P., Helmling, S., Stribling, D.S., Mazur, P., Ge, L., Wang, L., Klussmann, S., Macintyre, D.E., Howard, A.D., et al. (2006). Ghrelin neutralization by a ribonucleic acid-SPM ameliorates obesity in diet-induced obese mice. Endocrinology *147*, 1517–1526.

Shen, K., and Meyer, T. (1999). Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. Science *284*, 162–166.

Shen, L., Liang, F., Walensky, L.D., and Huganir, R.L. (2000). Regulation of AMPA receptor GluR1 subunit surface expression by a 4. 1N-linked actin cytoskeletal association. The Journal of Neuroscience□*20*, 7932–7940.

Sheng, M., and Kim, E. (2011). The postsynaptic organization of synapses. Cold Spring Harbor Perspectives in Biology *3*,.

Sheng, M., Malinow, R., and Huganir, R. (2013). Neuroscience: Strength in numbers. Nature *493*, 482–483.

Shepherd, J.D., and Huganir, R.L. (2007). The cell biology of synaptic plasticity: AMPA receptor trafficking. Annual Review of Cell and Developmental Biology *23*, 613–643.

Shi, L., Bian, X., Qu, Z., Ma, Z., Zhou, Y., Wang, K., Jiang, H., and Xie, J. (2013). Peptide hormone ghrelin enhances neuronal excitability by inhibition of Kv7/KCNQ channels. Nature Communications *4*, 1435.

Shi, S., Hayashi, Y., Esteban, J.A., and Malinow, R. (2001). Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. Cell *105*, 331–343.

Shi, S.H., Hayashi, Y., Petralia, R.S., Zaman, S.H., Wenthold, R.J., Svoboda, K., and Malinow, R. (1999). Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. Science *284*, 1811–1816.

Shibasaki, T., Yamauchi, N., Takeuchi, K., Ishii, S., Sugihara, H., and Wakabayashi, I. (1998). The growth hormone secretagogue KP-102-induced stimulation of food intake is modified by fasting, restraint stress, and somatostatin in rats. Neuroscience Letters *255*, 9–12.

Shiiya, T., Nakazato, M., Mizuta, M., Date, Y., Mondal, M.S., Tanaka, M., Nozoe, S.-I., Hosoda, H., Kangawa, K., and Matsukura, S. (2002). Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. The Journal of Clinical Endocrinology and Metabolism *87*, 240–244.

Siegel, G.J., Albers, R.W., Brady, S.T., and Price, D.L. (2006). Basic Neurochemistry, 7th edition.

Siekevitz, P. (1985). The postsynaptic density: a possible role in long-lasting effects in the central nervous system. Proceedings of the National Academy of Sciences of the United States of America *82*, 3494–3498.

Singh, L.P., Andy, J., Anyamale, V., Greene, K., Alexander, M., and Crook, E.D. (2001). Hexosamine-induced fibronectin protein synthesis in mesangial cells is associated with increases in cAMP responsive element binding (CREB) phosphorylation and nuclear CREB: the involvement of protein kinases A and C. Diabetes *50*, 2355–2362.

Sjöström, L., Lindroos, A.-K., Peltonen, M., Torgerson, J., Bouchard, C., Carlsson, B., Dahlgren, S., Larsson, B., Narbro, K., Sjöström, C.D., et al. (2004). Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery. The New England Journal of Medicine *351*, 2683–2693.

Skibicka, K.P., and Dickson, S.L. (2011). Ghrelin and food reward: the story of potential underlying substrates. Peptides *32*, 2265–2273.

Skibicka, K.P., Hansson, C., Egecioglu, E., and Dickson, S.L. (2012). Role of ghrelin in food reward: impact of ghrelin on sucrose self-administration and mesolimbic dopamine and acetylcholine receptor gene expression. Addiction Biology *17*, 95–107.

Small, C.J., and Bloom, S.R. (2004). Gut hormones and the control of appetite. Trends in Endocrinology and Metabolism *15*, 259–263.

Smart, T.G., and Paoletti, P. (2012). Synaptic neurotransmitter-gated receptors. Cold Spring Harbor Perspectives in Biology *4*,.

Smith, R., Feighner, S., Prendergast, K., Guan, X., and Howard, A. (1999). A New Orphan Receptor Involved in Pulsatile Growth Hormone Release. Trends in Endocrinology and Metabolism *10*, 128–135.

Smith, R.G. (2005). Development of growth hormone secretagogues. Endocrine Reviews *26*, 346–360.

Smith, R.G., Cheng, K., Schoen, W.R., Pong, S.S., Hickey, G., Jacks, T., Butler, B., Chan, W.W., Chaung, L.Y., and Judith, F. (1993). A nonpeptidyl growth hormone secretagogue. Science *260*, 1640–1643.

Smith, R.G., Jiang, H., and Sun, Y. (2005). Developments in ghrelin biology and potential clinical relevance. Trends in Endocrinology and Metabolism *16*, 436–442.

Smith, R.G., Leonard, R., Bailey, A.R., Palyha, O., Feighner, S., Tan, C., Mckee, K.K., Pong, S.S., Griffin, P., and Howard, A. (2001). Growth hormone secretagogue receptor family members and ligands. Endocrine *14*, 9–14.

Smith, R.G., Van der Ploeg, L.H., Howard, A.D., Feighner, S.D., Cheng, K., Hickey, G.J., Wyvratt, M.J., Fisher, M.H., Nargund, R.P., and Patchett, A.A. (1997). Peptidomimetic regulation of growth hormone secretion. Endocrine Reviews *18*, 621–645.

Smith, R.G., Sun, Y., Betancourt, L., and Asnicar, M. (2004). Growth hormone secretagogues: prospects and potential pitfalls. Best Practice & Research. Clinical Endocrinology & Metabolism *18*, 333–347.

Snyder, E.M., Philpot, B.D., Huber, K.M., Dong, X., Fallon, J.R., and Bear, M.F. (2001). Internalization of ionotropic glutamate receptors in response to mGluR activation. Nature Neuroscience *4*, 1079–1085.

Soares, J.-B., and Leite-Moreira, A.F. (2008). Ghrelin, des-acyl ghrelin and obestatin: three pieces of the same puzzle. Peptides *29*, 1255–1270.

Sobolevsky, A.I., Rosconi, M.P., and Gouaux, E. (2009). X-ray structure, symmetry and mechanism of an AMPA-subtype glutamate receptor. Nature *462*, 745–756.

Sommer, B., Köhler, M., Sprengel, R., and Seeburg, P.H. (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. Cell *67*, 11–19.

Soriano-Guillén, L., Barrios, V., Campos-Barros, A., and Argente, J. (2004). Ghrelin levels in obesity and anorexia nervosa: effect of weight reduction or recuperation. The Journal of Pediatrics *144*, 36–42.

Spencer, S.J., Xu, L., Clarke, M.A., Lemus, M., Reichenbach, A., Geenen, B., Kozicz, T., and Andrews, Z.B. (2012). Ghrelin regulates the hypothalamic-pituitary-adrenal axis and restricts anxiety after acute stress. Biological Psychiatry *72*, 457–465.

Stanley, B.G., and Leibowitz, S.F. (1984). Neuropeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. Life Sciences *35*, 2635–2642.

Strack, S., Choi, S., Lovinger, D.M., and Colbran, R.J. (1997). Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. The Journal of Biological Chemistry *272*, 13467–13470.

Stranahan, A.M., Norman, E.D., Lee, K., Cutler, R.G., Telljohann, R.S., Egan, J.M., and Mattson, M.P. (2008). Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. Hippocampus *18*, 1085–1088.

Sun, X., Zhao, Y., and Wolf, M.E. (2005). Dopamine receptor stimulation modulates AMPA receptor synaptic insertion in prefrontal cortex neurons. The Journal of Neuroscience *25*, 7342–7351.

Sun, Y., Ahmed, S., and Smith, R.G. (2003). Deletion of ghrelin impairs neither growth nor appetite. Molecular and Cellular Biology *23*, 7973–7981.

Sun, Y., Wang, P., Zheng, H., and Smith, R.G. (2004). Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proceedings of the National Academy of Sciences of the United States of America *101*, 4679–4684.

Swanson, G.T., Kamboj, S.K., and Cull-Candy, S.G. (1997). Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. The Journal of Neuroscience *17*, 58–69.

Taati, M., Nayebzadeh, H., and Zendehdel, M. (2011). The effects of DL-AP5 and glutamate on ghrelin-induced feeding behavior in 3-h food-deprived broiler cockerels. Journal of Physiology and Biochemistry *67*, 217–223.

Takahashi, T., Svoboda, K., and Malinow, R. (2003). Experience strengthening transmission by driving AMPA receptors into synapses. Science *299*, 1585–1588.

Takasaki, J., Saito, T., Taniguchi, M., Kawasaki, T., Moritani, Y., Hayashi, K., and Kobori, M. (2004). A novel Galphaq/11-selective inhibitor. The Journal of Biological Chemistry *279*, 47438–47445.

Takaya, K., Ariyasu, H., Kanamoto, N., Iwakura, H., Yoshimoto, A., Harada, M., Mori, K., Komatsu, Y., Usui, T., Shimatsu, A., et al. (2000). Ghrelin strongly stimulates growth hormone release in humans. The Journal of Clinical Endocrinology and Metabolism *85*, 4908–4911.

Tan, C.P., McKee, K.K., Liu, Q., Palyha, O.C., Feighner, S.D., Hreniuk, D.L., Smith, R.G., and Howard, A.D. (1998). Cloning and characterization of a human and murine T-cell orphan G-protein-coupled receptor similar to the growth hormone secretagogue and neurotensin receptors. Genomics *52*, 223–229.

Tanaka, M., Hayashida, Y., Iguchi, T., Nakao, N., Nakai, N., and Nakashima, K. (2001). Organization of the mouse ghrelin gene and promoter: occurrence of a short noncoding first exon. Endocrinology *142*, 3697–3700.

Tanaka, M., Naruo, T., Nagai, N., Kuroki, N., Shiiya, T., Nakazato, M., Matsukura, S., and Nozoe, S. (2003a). Habitual binge/purge behavior influences circulating ghrelin levels in eating disorders. Journal of Psychiatric Research *37*, 17–22.

Tanaka, M., Naruo, T., Yasuhara, D., Tatebe, Y., Nagai, N., Shiiya, T., Nakazato, M., Matsukura, S., and Nozoe, S. (2003b). Fasting plasma ghrelin levels in subtypes of anorexia nervosa. Psychoneuroendocrinology *28*, 829–835.

Tardin, C., Cognet, L., Bats, C., Lounis, B., and Choquet, D. (2003). Direct imaging of lateral movements of AMPA receptors inside synapses. The EMBO Journal *22*, 4656–4665.

Tauber, M., Conte Auriol, F., Moulin, P., Molinas, C., Delagnes, V., and Salles, J.P. (2004). Hyperghrelinemia is a common feature of Prader-Willi syndrome and pituitary stalk interruption: a pathophysiological hypothesis. Hormone Research *62*, 49–54.

Tena-Sempere, M., Barreiro, M.L., González, L.C., Gaytán, F., Zhang, F.-P., Caminos, J.E., Pinilla, L., Casanueva, F.F., Diéguez, C., and Aguilar, E. (2002). Novel expression and functional role of ghrelin in rat testis. Endocrinology *143*, 717–725.

Thaler, J.P., and Cummings, D.E. (2009). Minireview: Hormonal and metabolic mechanisms of diabetes remission after gastrointestinal surgery. Endocrinology *150*, 2518–2525.

Thomas, G.M., Lin, D.-T., Nuriya, M., and Huganir, R.L. (2008). Rapid and bi-directional regulation of AMPA receptor phosphorylation and trafficking by JNK. The EMBO Journal *27*, 361–372.

Thorner, M.O., Chapman, I.M., Gaylinn, B.D., Pezzoli, S.S., and Hartman, M.L. (1997). Growth hormone-releasing hormone and growth hormone-releasing peptide as therapeutic agents to enhance growth hormone secretion in disease and aging. Recent Progress in Hormone Research *52*, 215–44.

Tolle, V., Kadem, M., Bluet-Pajot, M.-T., Frere, D., Foulon, C., Bossu, C., Dardennes, R., Mounier, C., Zizzari, P., Lang, F., et al. (2003). Balance in ghrelin and leptin plasma levels in anorexia nervosa patients and constitutionally thin women. The Journal of Clinical Endocrinology and Metabolism *88*, 109–116.

Tomasetto, C., Karam, S.M., Ribieras, S., Masson, R., Lefèbvre, O., Staub, A., Alexander, G., Chenard, M.P., and Rio, M.C. (2000). Identification and characterization of a novel gastric peptide hormone: the motilin-related peptide. Gastroenterology *119*, 395–405.

Tomita, S., Adesnik, H., Sekiguchi, M., Zhang, W., Wada, K., Howe, J.R., Nicoll, R.A., and Bredt, D.S. (2005a). Stargazin modulates AMPA receptor gating and trafficking by distinct domains. Nature *435*, 1052–1058.

Tomita, S., Chen, L., Kawasaki, Y., Petralia, R.S., Wenthold, R.J., Nicoll, R.A., and Bredt, D.S. (2003). Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. The Journal of Cell Biology *161*, 805–816.

Tomita, S., Stein, V., Stocker, T.J., Nicoll, R. a, and Bredt, D.S. (2005b). Bidirectional synaptic plasticity regulated by phosphorylation of stargazin-like TARPs. Neuron *45*, 269–277.

Tong, J., Mannea, E., Aimé, P., Pfluger, P.T., Yi, C.-X., Castaneda, T.R., Davis, H.W., Ren, X., Pixley, S., Benoit, S., et al. (2011). Ghrelin enhances olfactory sensitivity and exploratory sniffing in rodents and humans. The Journal of Neuroscience *31*, 5841–5846.

Torsello, A., Ghe', C., Bresciani, E., Catapano, F., Ghigo, E., Deghenghi, R., Locatelli, V., and Muccioli, G. (2002). Short ghrelin peptides neither displace ghrelin binding in vitro nor stimulate GH release in vivo. Endocrinology *143*, 1968–1971.

Torsello, A., Luoni, M., Schweiger, F., Grilli, R., Guidi, M., Bresciani, E., Deghenghi, R., Müller, E.E., and Locatelli, V. (1998). Novel hexarelin analogs stimulate feeding in the rat through a mechanism not involving growth hormone release. European Journal of Pharmacology *360*, 123–129.

Torsello, A., Scibona, B., Leo, G., Bresciani, E., Avallone, R., Bulgarelli, I., Luoni, M., Zoli, M., Rindi, G., Cocchi, D., et al. (2003). Ontogeny and tissue-specific regulation of ghrelin mRNA expression suggest that ghrelin is primarily involved in the control of extraendocrine functions in the rat. Neuroendocrinology *77*, 91–99.

Toshinai, K., Mondal, M.S., Nakazato, M., Date, Y., Murakami, N., Kojima, M., Kangawa, K., and Matsukura, S. (2001). Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. Biochemical and Biophysical Research Communications *281*, 1220–1225.

Traynelis, S.F., Wollmuth, L.P., McBain, C.J., Menniti, F.S., Vance, K.M., Ogden, K.K., Hansen, K.B., Yuan, H., Myers, S.J., and Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation, and function. Pharmacological Reviews *62*, 405–496.

Tschöp, M., Smiley, D.L., and Heiman, M.L. (2000). Ghrelin induces adiposity in rodents. Nature *407*, 908–913.

Tschöp, M., Wawarta, R., Riepl, R.L., Friedrich, S., Bidlingmaier, M., Landgraf, R., and Folwaczny, C. (2001a). Post-prandial decrease of circulating human ghrelin levels. Journal of Endocrinological Investigation *24*, RC19–21.

Tschöp, M., Weyer, C., Tataranni, P.A., Devanarayan, V., Ravussin, E., and Heiman, M.L. (2001b). Circulating ghrelin levels are decreased in human obesity. Diabetes *50*, 707–709.

Ueki, N., Someya, K., Matsuo, Y., Wakamatsu, K., and Mukai, H. (2007). Cryptides: functional cryptic peptides hidden in protein structures. Biopolymers *88*, 190–198.

Ukkola, O. (2004). Peripheral regulation of food intake: new insights. Journal of Endocrinological Investigation *27*, 96–98.

Verdoorn, T.A., Burnashev, N., Monyer, H., Seeburg, P.H., and Sakmann, B. (1991). Structural determinants of ion flow through recombinant glutamate receptor channels. Science *252*, 1715–1718.

Vettor, R., Fabris, R., Pagano, C., and Federspil, G. (2002). Neuroendocrine regulation of eating behavior. Journal of Endocrinological Investigation *25*, 836–854.

Volante, M., Fulcheri, E., Allìa, E., Cerrato, M., Pucci, A., and Papotti, M. (2002). Ghrelin expression in fetal, infant, and adult human lung. The Journal of Histochemistry and Cytochemistry *50*, 1013–1021.

Volkow, N.D., Wang, G.-J., and Baler, R.D. (2011). Reward, dopamine and the control of food intake: implications for obesity. Trends in Cognitive Sciences *15*, 37–46.

Wajnrajch, M., Tem, I., Gertner, J., and Leibel, R. (2000). Genomic Organization of the Human GHRELIN Gene. Journal of Endocrine Genetics *1*, 231–233.

Wang, H.-J., Geller, F., Dempfle, A., Schäuble, N., Friedel, S., Lichtner, P., Fontenla-Horro, F., Wudy, S., Hagemann, S., Gortner, L., et al. (2004). Ghrelin receptor gene: identification of several sequence variants in extremely obese children and adolescents, healthy normal-weight and underweight students, and children with short normal stature. The Journal of Clinical Endocrinology and Metabolism *89*, 157–162.

Watkins, J.C., Davies, J., Evans, R.H., Francis, A.A., and Jones, A.W. (1981). Pharmacology of receptors for excitatory amino acids. Advances in Biochemical Psychopharmacology *27*, 263–273.

Watkins, J.C., and Jane, D.E. (2006). The glutamate story. British Journal of Pharmacology *147 Suppl* , S100–8.

Wegener, G., Volke, V., and Rosenberg, R. (2000). Endogenous nitric oxide decreases hippocampal levels of serotonin and dopamine in vivo. British Journal of Pharmacology *130*, 575–580.

Wei, W., Wang, G., Qi, X., Englander, E.W., and Greeley, G.H. (2005). Characterization and regulation of the rat and human ghrelin promoters. Endocrinology *146*, 1611–1625.

Wenthold, R.J., Petralia, R.S., Blahos J, I.I., and Niedzielski, A.S. (1996). Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. The Journal of Neuroscience *16*, 1982–1989.

Whitlock, J.R., Heynen, A.J., Shuler, M.G., and Bear, M.F. (2006). Learning induces long-term potentiation in the hippocampus. Science *313*, 1093–1097.

Wise, A., Jupe, S.C., and Rees, S. (2004). The identification of ligands at orphan G-protein coupled receptors. Annual Review of Pharmacology and Toxicology *44*, 43–66.

Wisse, B.E., Frayo, R.S., Schwartz, M.W., and Cummings, D.E. (2001). Reversal of cancer anorexia by blockade of central melanocortin receptors in rats. Endocrinology *142*, 3292–3301.

Witte, A. V, Fobker, M., Gellner, R., Knecht, S., and Flöel, A. (2009). Caloric restriction improves memory in elderly humans. Proceedings of the National Academy of Sciences of the United States of America *106*, 1255–1260.

Wo, Z.G., and Oswald, R.E. (1995). Unraveling the modular design of glutamate-gated ion channels. Trends in Neurosciences *18*, 161–168.

Wollmuth, L.P., and Traynelis, S.F. (2009). Neuroscience: Excitatory view of a receptor. Nature *462*, 729–731.

Wortley, K.E., Anderson, K.D., Garcia, K., Murray, J.D., Malinova, L., Liu, R., Moncrieffe, M., Thabet, K., Cox, H.J., Yancopoulos, G.D., et al. (2004). Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. Proceedings of the National Academy of Sciences of the United States of America *101*, 8227–8232.

Wren, A.M., Seal, L.J., Cohen, M.A., Brynes, A.E., Frost, G.S., Murphy, K.G., Dhillo, W.S., Ghatei, M.A., and Bloom, S.R. (2001a). Ghrelin enhances appetite and increases food intake in humans. The Journal of Clinical Endocrinology and Metabolism *86*, 5992.

Wren, A.M., Small, C.J., Abbott, C.R., Dhillo, W.S., Seal, L.J., Cohen, M.A., Batterham, R.L., Taheri, S., Stanley, S.A., Ghatei, M.A., et al. (2001b). Ghrelin causes hyperphagia and obesity in rats. Diabetes *50*, 2540–2547.

Wren, A.M., Small, C.J., Ward, H.L., Murphy, K.G., Dakin, C.L., Taheri, S., Kennedy, A.R., Roberts, G.H., Morgan, D.G., Ghatei, M.A., et al. (2000). The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology *141*, 4325–4328.

Wu, A., Molteni, R., Ying, Z., and Gomez-Pinilla, F. (2003). A saturated-fat diet aggravates the outcome of traumatic brain injury on hippocampal plasticity and cognitive function by reducing brain-derived neurotrophic factor. Neuroscience *119*, 365–375.

Wu, G., Malinow, R., and Cline, H.T. (1996). Maturation of a central glutamatergic synapse. Science *274*, 972–976.

Wynne, K., Stanley, S., and Bloom, S. (2004). The gut and regulation of body weight. The Journal of Clinical Endocrinology and Metabolism *89*, 2576–2582.

Xiao, M.Y., Zhou, Q., and Nicoll, R.A. (2001). Metabotropic glutamate receptor activation causes a rapid redistribution of AMPA receptors. Neuropharmacology *41*, 664–671.

Xu, X., Jhun, B.S., Ha, C.H., and Jin, Z.-G. (2008). Molecular mechanisms of ghrelin-mediated endothelial nitric oxide synthase activation. Endocrinology *149*, 4183–4192.

Yabuki, A., Ojima, T., Kojima, M., Nishi, Y., Mifune, H., Matsumoto, M., Kamimura, R., Masuyama, T., and Suzuki, S. (2004). Characterization and species differences in gastric ghrelin cells from mice, rats and hamsters. Journal of Anatomy *205*, 239–246.

Yamamoto, D., Ikeshita, N., Daito, R., Herningtyas, E.H., Toda, K., Takahashi, K., Iida, K., Takahashi, Y., Kaji, H., Chihara, K., et al. (2007). Neither intravenous nor intracerebroventricular administration of obestatin affects the secretion of GH, PRL, TSH and ACTH in rats. Regulatory Peptides *138*, 141–144.

Yang, J., Brown, M.S., Liang, G., Grishin, N. V, and Goldstein, J.L. (2008). Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell *132*, 387–396.

Yeh, A.H., Jeffery, P.L., Duncan, R.P., Herington, A.C., and Chopin, L.K. (2005). Ghrelin and a novel preproghrelin isoform are highly expressed in prostate cancer and ghrelin activates mitogen-activated protein kinase in prostate cancer. Clinical Cancer Research□*11*, 8295–8303.

Yin, X., Li, Y., Xu, G., An, W., and Zhang, W. (2009). Ghrelin fluctuation, what determines its production? Acta Biochimica Et Biophysica Sinica *41*, 188–197.

Yudowski, G.A., Puthenveedu, M.A., Leonoudakis, D., Panicker, S., Thorn, K.S., Beattie, E.C., and Von Zastrow, M. (2007). Real-time imaging of discrete exocytic events mediating surface delivery of AMPA receptors. The Journal of Neuroscience *27*, 11112–11121.

Zamanillo, D., Sprengel, R., Hvalby, O., Jensen, V., Burnashev, N., Rozov, A., Kaiser, K.M., Köster, H.J., Borchardt, T., Worley, P., et al. (1999). Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. Science *284*, 1805–1811.

Zhang, J. V, Ren, P.-G., Avsian-Kretchmer, O., Luo, C.-W., Rauch, R., Klein, C., and Hsueh, A.J.W. (2005). Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science *310*, 996–999.

Zhang, W., Lin, T.R., Hu, Y., Fan, Y., Zhao, L., Stuenkel, E.L., and Mulholland, M.W. (2004). Ghrelin stimulates neurogenesis in the dorsal motor nucleus of the vagus. The Journal of Physiology *559*, 729–737.

Zhang, Y.-P., Holbro, N., and Oertner, T.G. (2008). Optical induction of plasticity at single synapses reveals input-specific accumulation of alphaCaMKII. Proceedings of the National Academy of Sciences of the United States of America *105*, 12039–12044.

Zhao, Z., Sakata, I., Okubo, Y., Koike, K., Kangawa, K., and Sakai, T. (2008). Gastric leptin, but not estrogen and somatostatin, contributes to the elevation of ghrelin mRNA expression level in fasted rats. The Journal of Endocrinology *196*, 529–538.

Zhu, J.J., Esteban, J.A., Hayashi, Y., and Malinow, R. (2000). Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. Nature Neuroscience *3*, 1098–1106.

Zhu, J.J., and Malinow, R. (2002). Acute versus chronic NMDA receptor blockade and synaptic AMPA receptor delivery. Nature Neuroscience *5*, 513–514.

Zhu, J.J., Qin, Y., Zhao, M., Van Aelst, L., and Malinow, R. (2002). Ras and Rap control AMPA receptor trafficking during synaptic plasticity. Cell *110*, 443–455.

Zhu, X., Cao, Y., Voogd, K., Voodg, K., and Steiner, D.F. (2006). On the processing of proghrelin to ghrelin. The Journal of Biological Chemistry *281*, 38867–38870.

Ziff, E.B. (2007). TARPs and the AMPA receptor trafficking paradox. Neuron *53*, 627–633.

Zigman, J.M., Jones, J.E., Lee, C.E., Saper, C.B., and Elmquist, J.K. (2006). Expression of ghrelin receptor mRNA in the rat and the mouse brain. The Journal of Comparative Neurology *494*, 528–548.

Zorrilla, E.P., Iwasaki, S., Moss, J.A., Chang, J., Otsuji, J., Inoue, K., Meijler, M.M., and Janda, K.D. (2006). Vaccination against weight gain. Proceedings of the National Academy of Sciences of the United States of America *103*, 13226–13231.