DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Analysis of the Ghrelin/GHSR1 system in dentate gyrus neural progenitor cells

Irina Suheila Martins Leal Fonseca

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Abbreviations

BBB	blood brain barrier
BSA	bovine serum albumin
AHP	adult hippocampal progenitor
CA1	Corunu Ammonis area 1 from hippocampus
CA3	Corunu Ammonis area 3 from hippocampus
CNS	central nervous system
CGRP	calcitonin gene-related peptide
CVO	circumventricular organs
DCX	doublecortin
DG	dentate gyrus
DIV	days *in vitro*
ER	endoplasmic reticulum
GFAP	glial fibrilary acidic protein
GH	growth hormone
GHSR1	growth hormone secretagogue receptor type 1
GHSR-1a	growth hormone secretagogue receptor type 1a
GHSR-1b	growth hormone secretagogue receptor type 1b
GPR39	G-protein coupled receptor 39
GSK3β	glycogen synthase kinase-3β
GOAT	ghrelin o-acyl transferase
IGF-1	insulin-like growth factor-1
Jak2/ STAT3	janus kinase 2/ signal transducer and activator of transcription 3
MAP2	microtubule-associated protein 2
MEK/ERK1/2	mitogen-activated protein kinase/ extracellular signal- regulated kinases 1/2
mTOR/ $p70^{s6k}$	mammalian target ofrapamycin/ ribosomal protein S6 kinase
NeuN	neuronal nuclear antigen (FOX-3)
NPCs	neural progenitor cells
NSCs	neural stem cells
p13/AKT	p13-kinase/ protein kinase B
RMS	rostral migratory stream
ROIs	region of interest
SDR	spontaneous dwarf rats
SGZ	subgranular zone
SN	substancia nigra
Sox2	sex determining region Y (SRY)-box 2
SVZ	subventricular zone
TDM	trans-membrane domain
VTA	ventral tegmental area

Resumo

Ao longo da vida, novos neurónios são formados a partir de células progenitoras neurais (CPN). No cérebro adulto, dois principais nichos neurogénicos foram descritos: a zona subventricular (ZSV) dos ventrículos laterais e a zona subgranular (ZSG) do giro dentado (GD). Os neurónios recém formados do GD integram-se numa circuito funcional pré-estabelecido, tendo um papel no funcionamento da memória hipocampal. A produção desses novos neurónios é regulada por vários factores relacionados com o estilo de vida, incluindo hábitos alimentares. A grelina, uma hormona peptídea envolvida na regulação do apetite, tem sido descrita a aumentar a proliferação de CPN do hipocampo de ratos adultos e a modular a memoria. Contudo, ainda há controvérsias sobre a capacidade da grelina exercer um efeito direto nas CPN e células derivadas das CPN através da sua ligação ao seu receptor: o receptor secretagogo da hormona de crescimento 1a, GHSR-1a.

A nossa hipótese de trabalho considera que o sistema da grelina e o seu receptor exerce um efeito importante e crucial sobre a sobrevivência, maturação e diferenciação de neurónios derivados das células progenitores neurais do hipocampo. Os nossos resultados mostram que o receptor da grelina, GHSR-1a, está ausente em culturas de células derivadas das neuroesferas do giro dentado. No entanto, observamos que uma outra isoforma do GHSR1 (provavelmente a sua forma truncada, GHSR-1b) é expressa por células neuroprecursoras e neurónios em diferenciação. Além disso, fornecemos pela primeira vez, evidencias da produção de grelina em culturas de neuroesferas do giro dentado, e observamos que a adição de grelina exógena acylada nas nossas culturas não tem nenhum efeito na proliferação ou diferenciação neuronal das CPN do GD.

Os nossos dados demonstram que o sistema da grelina está presente em culturas celulares de CPN do GD, sugerindo que este sistema pode desempenhar um papel direto na modulação das CPNs e células derivadas do GD.

Palavras-chave: Neurogénese no hipocampo adulto, giro dentado (GD), sistema da Grelina/GHSR-1, receptor secretagogo da hormona de crescimento 1 (GHSR1), Células progenitoras neurais (CPN).

Abstract

Throughout life, new neurons are formed from neural progenitor cells (NPCs). In the adult brain, two main neurogenic niches have been described: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG). DG newly-generated neurons integrate into a pre-established and functional network, having a role on hippocampal memory function. The production of these new neurons is regulated by several lifestyle-related factors, including dietary habits. Ghrelin, a peptide hormone that is involved in the regulation of appetite, has been described to increase proliferation of adult rat hippocampal NPCs and to modulate memory. However, there is still some controversy about whether ghrelin is able to exert a direct effect on NPCs and NPC derived cells by binding to its receptor: the growth hormone secretagogue receptor 1a, GHSR-1a.

In this work, we hypothesize that Ghrelin/GHSR-1a system exerts a crucial and direct effect on the survival, maturation and differentiation of neurons derived from hippocampal neural progenitor cells (NPCs). We show that GHSR-1a is absent in neurosphere cell cultures derived from the dentate gyrus (DG). Nevertheless, we observed that another isoform of GHSR1 (probably its truncated isoform GHSR1b) is expressed by neuroprecursor cells (NPCs) and differentiating neurons. Furthermore, we provide, for the first time, evidences of the production of ghrelin in DG neurosphere cultures. Finally, we observed that the addition of exogenous acylated ghrelin to our cultures has no effect on cell proliferation or neuronal differentiation of DG NPCs.

Our data demonstrate that ghrelin/GHSR1 system is present in DG derived neurosphere cell cultures, suggesting that this system may play a direct role in the modulation of DG NPCs and derived cells.

Keywords: Adult hippocampal neurogenesis, dentate gyrus (DG), Ghrelin/GHSR-1 system, Growth Hormone Secretagogue Receptor type 1 (GHSR-1), Neural progenitor cells (NPCs).

Chapter 1

Introduction

Caloric restriction presents wide-range health benefits, e.g. protects against age-related neuronal loss and neurodegenerative disorders like Alzheimer's disease (Gillette-Guyonnet and Vellas 2008; Francis and Stevenson 2013). However, the mechanisms involved in the protection of central nervous system (CNS) mediated by caloric restriction are not fully understood. It has been described that caloric restriction has an important role in the regulation of adult neural stem cells, increasing adult neurogenesis in young adult rats and reducing age-related neurogenesis decline in older animals (Lee, Seroogy, and Mattson 2002; Bondolfi et al. 2004; Park et al. 2013). These diet-related effects on adult neurogenesis have been proposed as one of the mechanisms by which dietary habits affect CNS function (Levenson and Rich 2007; Maalouf, Rho, and Mattson 2009).
Ghrelin is the only known and established orexigenic appetite stimulant gut hormone (Kojima et al. 1999; Y Date et al. 2000; Takaya et al. 2000). High-fat diets induce a decrease in ghrelin levels (Beck, Musse, and Stricker-Krongrad 2002; Lomenick et al. 2009) and an impairment on hippocampus-dependent synaptic plasticity and spatial memory (Wu et al. 2003; Stranahan et al. 2008). On the other hand, caloric restriction leads to an increase in ghrelin levels (Cummings et al. 2002; Weigle et al. 2003; Leidy et al. 2007; Yang et al. 2007), promoting learning consolidation and facilitating synaptic plasticity (Fontán-Lozano et al. 2007; Witte et al. 2009). Interestingly, the factors that modulate ghrelin levels are also known to affect adult neurogenesis. Indeed, it has been described that ghrelin increases dentate gyrus (DG) adult neurogenesis, enhancing learning and memory in rodents (Moon et al. 2009; Endan Li et al. 2013; Z. Zhao et al. 2014). Considering that neurogenesis in the DG has been proposed to mediate hippocampal-dependent learning and memory (C. Zhao, Deng, and Gage 2008a), the ghrelin-induced stimulation of neurogenesis in hippocampal NPCs may play an important role in the ability of ghrelin to enhance memory performance. Despite ghrelin is a mediator of the effects of diet on adult neurogenesis and memory function, there are some uncertainties about how ghrelin exert its modulation. Therefore, in this work we aimed to deeply explore the role of ghrelin/ghrelin receptor system on the regulation of DG neural progenitor cells (NPCs) and production of new neurons.

Ghrelin

Ghrelin is a twenty eight-amino acid peptide hormone (Fig. 1A) that was isolated from the rat and human stomach in 1999 by Kojima et al. (Kojima et al. 1999). It is abundantly produced from endocrine cells in the gastrointestinal mucosa (Sakata and Sakai 2010) and other peripheral tissues, like the pancreas, ovary and adrenal cortex (Klok, Jakobsdottir, and Drent 2007). Ghrelin is derived from the proteolytic cleavage of pre-pro-ghrelin and pro-ghrelin (Fig. 1B), and only exerts its orexigenic effect when it is acylated. The enzyme Ghrelin O-acyl transferase (GOAT) links an octanoyl group post-translationaly to serine at position 3, forming the acylated form of ghrelin. Acylation of ghrelin is important for ghrelin activation and binding to its receptor, the growth hormone secretagogue receptor 1a (GHSR-1a). However, unacylated-ghrelin, is the predominant form of circulating ghrelin in the bloodstream (Y Date et al. 2000; Hosoda et al. 2000; Romero et al. 2010; Kojima and Kangawa 2005) and has various physiological functions through a yet undetermined receptor. Moreover, in 2005 Obestatin, a 23 amino-acid peptide, was identified as another hormone derived from pre-pro-ghrelin (Fig. 1B) (Zhang et al. 2005; Chartrel et al. 2007; Tang et al. 2008).

Obestatin has been described as a ligand of the orphan G-protein-coupled receptor, GPR39, a member of the ghrelin receptor family. However, this finding has been questioned, and it still remains unclear and a matter of debate (Lauwers et al. 2006; Chartrel et al. 2007; Holst et al. 2007).

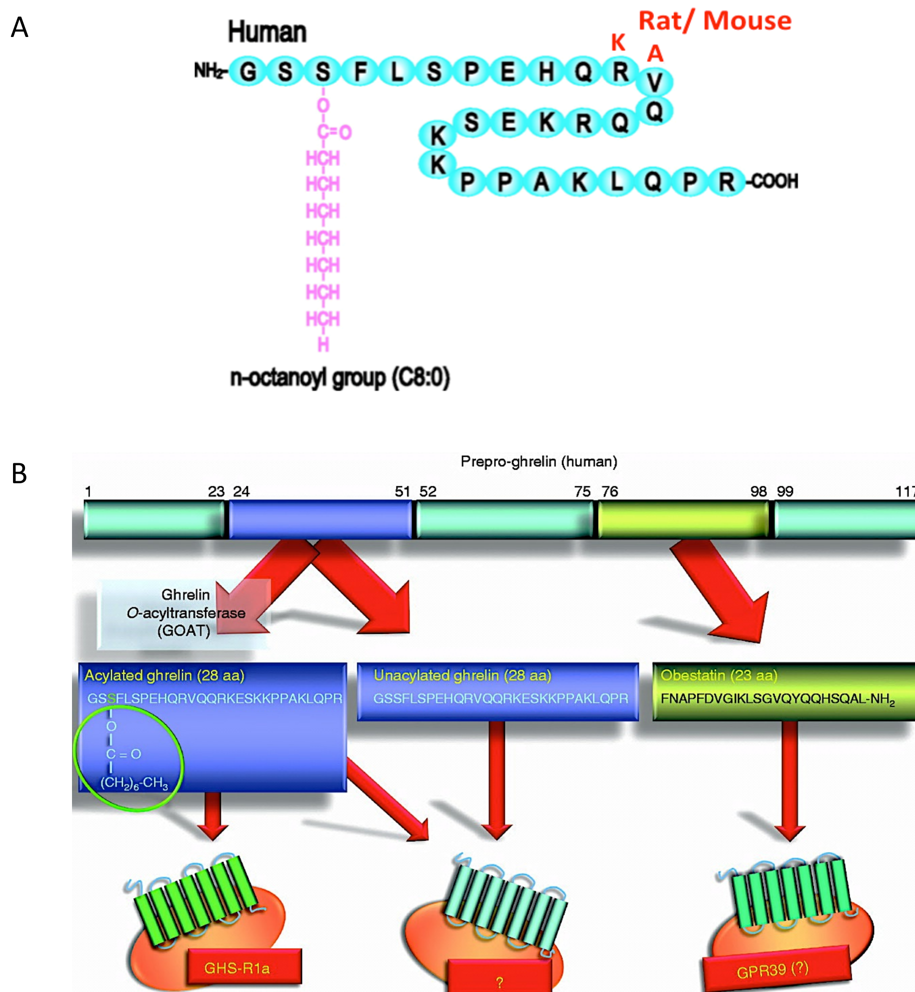


Figure 1.1: (A) Human and rat/mouse ghrelins differ only in two amino acid residues, having (in the three species) a 28-amino acid sequence and being acylated on Serine 3. (B) Ghrelin derives from the cleavage of the N-terminal fragment of pre-pro-ghrelin, while obestatin originates from the C-terminal fragment of this precursor. Ghrelin exists in two main forms: acylated and unacylated. Ghrelin acylation on serine 3, promoted by GOAT, is essential for its binding to GHSR-1a and for endocrine functions. Unacylated ghrelin lacks the capacity to bind to GHSR-1a and its receptor remains unidentified. Obestatin was proposed as the natural ligand of the GPR39 orphan receptor but this possibility still needs to be demonstrated. GHSR-1a, Growth Hormone secretagogue receptor type 1a; GOAT, ghrelin O-acyltransferase; GPR39, G-protein-coupled receptor 39. (A) Adapted from (Kojima and Kangawa 2005) and (B) from (Granata et al. 2010).

Ghrelin has multiple functions, including regulation of growth hormone (GH) release, energy homeostasis and appetite stimulation (table 1.1) (Hubina, Góth, and Korbonits 2005; Kojima and Kangawa 2005; Gil-Campos et al. 2006). Ghrelin is mainly produced in the stomach released to the

bloodstream, where it reaches a maximum before the onset of each meal. Ghrelin binds to its receptor in the hypothalamus promoting hunger. Finally, after food ingestion, plasma levels of ghrelin diminish leading to a decrease of appetite (Fig. 1.2). Korbonits et al. suggested that ghrelin might reach the CNS in three different ways: by crossing the Blood-Brain Barrier (BBB), through the vagal nerve and nucleus tractus solitarus or being produced locally in the hypothalamus (Korbonits et al. 2004). Indeed, it has been previously reported that ghrelin crosses the BBB and enter the hippocampus (Banks et al. 2002; Diano et al. 2006). Furthermore, the induction of GH release and appetite stimulation after ghrelin intravenous injection is dramatically decreased when the vagal nerve is cut, suggesting that the vagal nerve is required for the stimulatory effect of ghrelin (Yukari Date et al. 2002; Williams et al. 2003). Finally, there are also evidences of local synthesis of ghrelin in the hypothalamus (Lu et al. 2002; Cowley et al. 2003). Therefore, it is possible that ghrelin reaches the brain in multiple ways. Importantly, it is perfectly know that once into the brain, ghrelin exerts it effects by binding to its receptor, the growth hormone secretagogue receptor 1a (GHSR-1a) (Kojima et al. 1999; Takaya et al. 2000). Indeed, GHSR-1a is the only functional ghrelin receptor characterized (Howard et al. 1996) and identified until now.

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.

Table 1.1: Multiple physiological functions of ghrelin in human and/or rats. Adapted from (Sato et al. 2012).

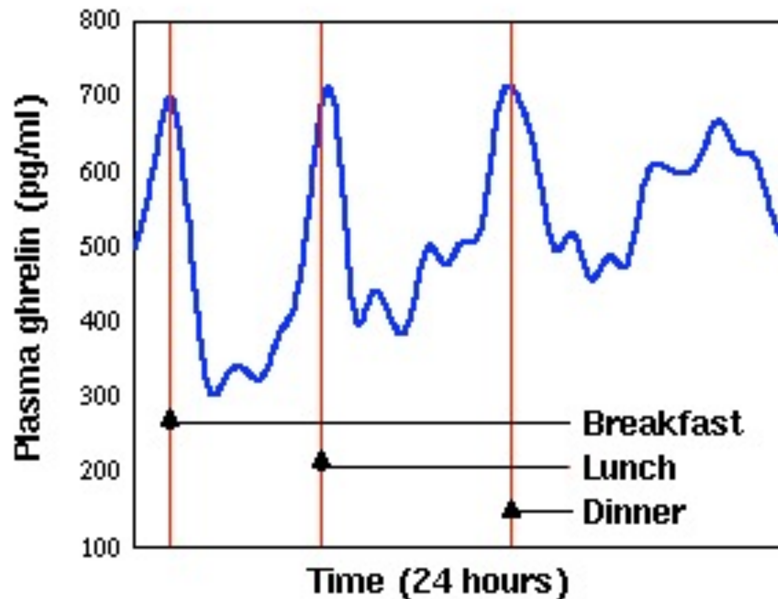


Figure 1.2: Representative 24h profile of plasma ghrelin levels from a subject consuming breakfast, lunch and dinner. Plasma ghrelin level peaks before the onset of each meal and decreases within 1h after food intake. Adapted from (Cummings et al. 2001).

Growth Hormone Secretagogue Receptor 1 (GHSR-1)

The human Growth hormone secretagogue receptor gene is located on chromosome 3q26 and encodes a G-protein coupled 7 trans-membrane receptor (Liu, Garcia, and Korbonits 2011). The gene consists of two exons and a single intron. The first exon encodes the trans-membrane domains (TMD) 1-5, while the second exon encodes the TMD 6 and 7 (Howard et al. 1996). Two splice variants originate from this gene: 1a and 1b. *GHSR-1a* transcript is constituted by the two exons that encode for the functional receptor with seven TMD and 366 amino acids. The second transcript, *GHSR-1b*, retains part of the intronic sequence and does not function as a receptor for ghrelin (Petersenn et al. 2001). GHSR-1b is described as a truncated isoform of ghrelin receptor, with five TMD and 289 amino acids (Fig. 1.3). GHSR-1b is expressed in various tissues (Gnanapavan et al. 2002), so it is possible that this receptor has an unidentified biological function. Indeed it has been described that GHSR-1b forms heterodimers with ghrelin receptor, decreasing GHSR-1a cell surface expression by retaining it in the Endoplasmic reticulum (ER) (Leung et al. 2007; Chow et al. 2012; Kit-Man Chu et al. 2007). Therefore, GHSR-1b may act as an endogenous modulator of GHSR-1a. Despite rat *Ghsr1* gene and splice variants are similar to the human ones, mouse variants have not been described. In results section we propose a model based on the comparison of mouse *Ghsr1* gene, mouse *Ghsr-1a* mRNA and known data from human and rat *Ghsr1* genes and mRNA splice variants.

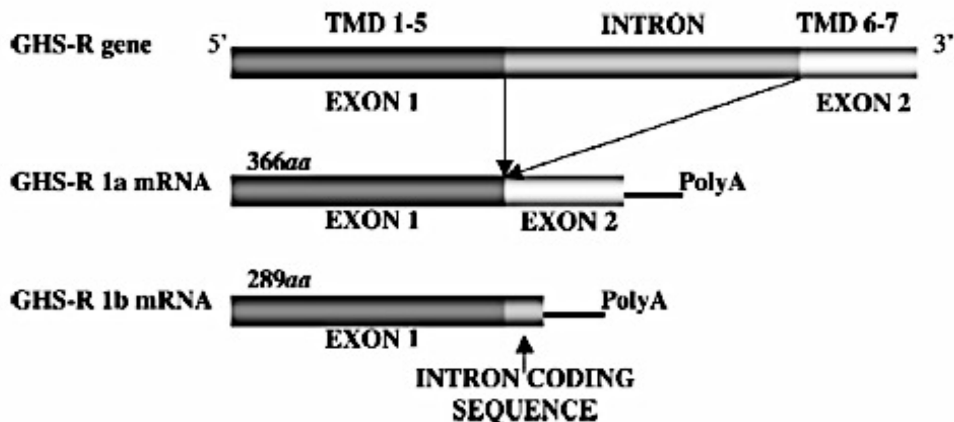


Figure 1.3: The growth hormone secretagogue receptor human gene and mRNA variants. The full length *GHSR-1a* mRNA encodes seven TMD from exon one and two. The truncated mRNA variant, *GHSR-1b*, retains part of the intronic sequence and encodes a five TMD protein with a unique C-terminus. GHSR, Ghrowth hormone secretagogue receptor; TMD, Trans-membrane domain. Adapted from (Jeffery, Herington, and Chopin 2003).

GHSR-1a is expressed in numerous peripheral tissues, including stomach, pancreas, spleen, thyroid, adrenal glands, heart, and also in the CNS. Curiously, GHSR-1b is more widely distributed then GHSR-1a, being also present in liver, lung, bladder, prostate, adipose tissue and esophagus, among others (Gnanapavan et al. 2002). In the CNS, GHSR-1a and GHSR-1b are found at highest concentrations in the pituitary and hypothalamus (Howard et al*.* 1996; Gnanapavan et al*.* 2002). Interestingly, GHSR-1a is also expressed in extra-hypothalamic regions (Guan et al. 1997; Zigman et al. 2006), like the ventral tegmental area (VTA), substancia nigra (SN), dorsal raphe nucleus and the hippocampus (Albarrán-Zeckler and Smith 2013). In the mouse hippocampus, GHSR-1a is found in the CA1 and CA3 regions (Diano et al. 2006), whereas there are no data about GHSR-1b in these regions. Importantly, ghrelin also exerts a role in CNS extra-hypothalamic regions, controling several brain functions. Indeed, as previously mentioned, ghrelin has been shown to hippocampal neurogenesis (Moon et al. 2009; Endan Li et al. 2013; Z. Zhao et al. 2014).

Adult Neurogenesis

Neurogenesis is the process of generating new functional neurons from neural stem and progenitor cells. This process persists into adulthood, and occurs mainly in two brain regions: the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the DG of the hippocampus(Fig. 1.4) (Altman 1962; Eriksson et al. 1998; Alvarez-Buylla and Garcia-Verdugo 2002). In the SVZ of the lateral ventricles and in the SGZ of DG there are neural stem cells that divide asymmetrically and generate NPCs. These NPCs proliferate and then differentiate into neurons, astrocytes and oligodendrocytes (Fig. 1.5) (Gage 2000). In rodents, neuroblasts from the SVZ migrate over a great distance through the rostral migratory stream (RMS) and become granule and periglomerular neurons in the olfactory bulb, whereas neuroblasts from the SGZ of the DG migrate into the granule cell layer and become dentate granule cells (C. Zhao, Deng, and Gage 2008b). New born neurons of the DG integrate into pre-established hippocampal circuitry and play an important role in learning and memory (Eriksson et al. 1998; Garthe, Behr, and Kempermann 2009; Piatti, Ewell, and Leutgeb 2013).

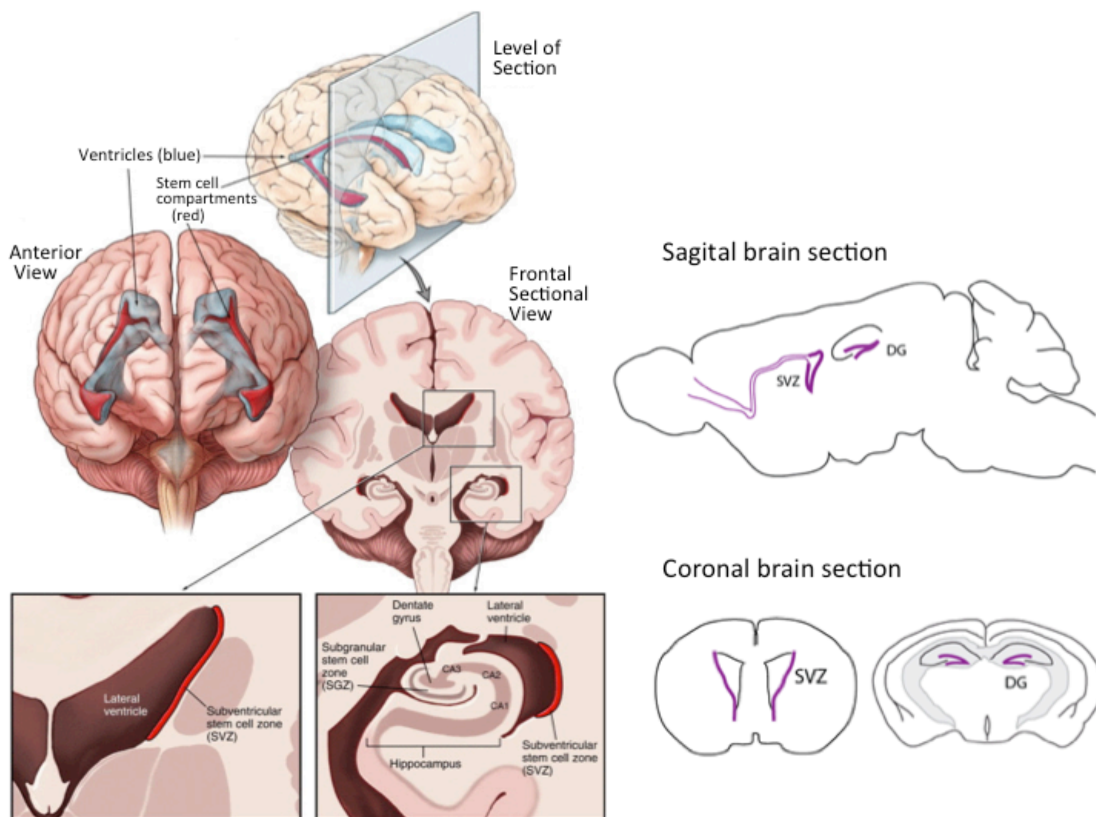


Figure 1.4: Neurogenic niches in adult human (left) and mouse (right) brain. SVZ of the lateral ventricles and SGZ of DG in the Hippocampus are the main regions where adult neurogenesis occurs. DG, Dentate gyrus; SGZ, Subgranular zone; SVZ, Subventricular zone. Adapted from https://beyondthedish.files.wordpress.com/2014/02/subventricular-zone.gif and (Seib, D & Martin-Villalba, A 2013).

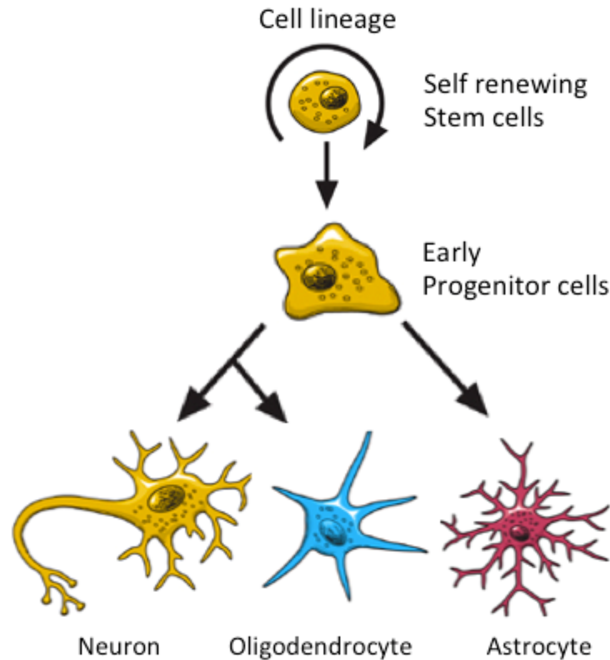


Figure 1.5: NPCs differentiation during the process of neurogenesis. Adapted from (Morrison and Hof 1997).

The process of neurogenesis can be modulated by different life factors (Tanti et al. 2012). For example, stress decreases adult neurogenesis in the hippocampus (Mirescu and Gould 2006; Schoenfeld and Gould 2012) while enriched environment, exercise and hypocaloric diet increase adult hippocampal neurogenesis (Lee, Seroogy, and Mattson 2002; Brown et al. 2003; Olson et al. 2006). It is well know that caloric restriction enhance neurogenesis in the adult DG (Levenson and Rich 2007; Stangl and Thuret 2009), however, the molecular mechanism is not well known. Ghrelin, which levels are increased in hypocaloric diets, has also been shown to have an effect in adult hippocampal neurogenesis (Moon et al. 2009; Endan Li et al. 2012; Endan Li et al. 2013; Moon, Cha, and Mook-Jung 2014; Z. Zhao et al. 2014). Therefore, the effect of diet on hippocampal neurogenesis might be mediated by ghrelin action in the DG neurogenic niche.

Ghrelin/GHSR-1 system and adult neurogenesis

Ghrelin has been described to modulate hippocampal neurogenesis by increasing cell proliferation (Moon et al. 2009; Endan Li et al. 2012; Endan Li et al. 2013; Moon, Cha, and Mook-Jung 2014; Z. Zhao et al. 2014). Indeed, Chung et al. showed that ghrelin exerts its proliferative effect in neural stem cells (NSCs) of the hippocampus by activating the MEK/ ERK1/2, P13/Akt and Jak2/STAT3 signaling pathways (Chung et al. 2013). These signaling pathways are known to be involved in several cellular processes, such as cell proliferation, differentiation, survival and apoptosis (Leonard and O'Shea 1998; Hers, Vincent, and Tavaré 2011; Roskoski 2012). The proliferative effect of ghrelin in NPCs is thought to be mediated through its receptor, the GHSR-1a. *In vivo*, GHSR-1a has been detected by immunofluorescence in the SVZ and in the SGZ of the DG, where ghrelin has been described to promote cell proliferation and neuronal differentiation (Endan Li et al. 2013; E Li et al.

2014). However, it is controversial whether ghrelin receptor is present on hippocampal NPCs. Till our knowledge, two studies have analyzed the effect of ghrelin in cell cultures of hippocampal NPCs. Johansson et al*.* showed by qPCR, using rat GHSR-1a specific primers (targeting exon 1 and 2), that there is no expression of ghrelin receptor in adult rat hippocampal progenitor (AHP) cells (Johansson et al. 2008). Nevertheless, Chung et al. described the presence of GHSR-1a in adult rat hippocampal NSCs using western blotting and immunocytochemistry (Chung et al. 2013). Despite of the contradictory data about the presence of GHSR-1a, both studies showed that ghrelin increases cell proliferation in NPCs cultures (Johansson et al. 2008; Chung et al. 2013). However, whether the effect of ghrelin on NPCs is direct or indirect is also a matter of discussion. It is known that ghrelin potently stimulate GH secretion from the pituitary and consequently increases insulin-like growth factor-1 (IGF-1) production, which is known to increase neurogenesis (Torres-Aleman 2010). Li et al. demonstrate that peripheral administration of ghrelin increased cell proliferation and neuronal differentiation in the SGZ of spontaneous dwarf rats (SDR) that show total loss of circulating GH, suggesting that ghrelin regulates adult hippocampal neurogenesis independently of GH/IGF-1 axis (Endan Li et al. 2013). However, another study suggested that the effects of ghrelin on neurogenesis are mediated by a different indirect pathway in which ghrelin may stimulate neurons in circumventricular organs (CVOs), increasing calcitonin gene-related peptide (CGRP) release in the hippocampus, which in turn increase IGF-I production and, therefore, adult hippocampal neurogenesis (Chen 2012).

It is still not clear whether the effect of ghrelin in NPCs is directly mediated by its binding to GHSR-1a, and most of the works focused on the effect of ghrelin in NPCs proliferation. *In vitro* culture of adult hippocampal neural precursor cells is a key tool for deciphering the cellular and molecular mechanisms that regulate adult neurogenesis. On the present study, we took advantage of cell cultures of DG derived neurospheres, which contains NPCs, to study the presence and regulatory role of ghrelin/GHSR1 system on precursor cells independently of exogenous niche signals.

Objectives and Hypothesis

The present work bases on the hypothesis that Ghrelin/GHSR-1a system exerts a crucial and direct effect on the survival, maturation and differentiation of neurons derived from hippocampal neural progenitor cells (NPCs).

To test our hypothesis, we have two main objectives:

- To analyze the expression of ghrelin and growth hormone secretagogue receptor 1 in dentate gyrus neurosphere cell cultures.

- To investigate the role of ghrelin/GHSR-1 system on cell proliferation and neuronal differentiation by treating NPC cultures with ghrelin.

Investigating the presence and function of Ghrelin/GHSR-1 system in dentate gyrus neural progenitor cells, we hope to provide new evidences about its direct role on the modulation of adult neurogenesis.

Chapter 2

Methodology

2.1 Cell culture

Neurospheres were obtained from the dentate gyrus (DG) of postnatal 3-days-old (P3) C57BL/6J mice by dissociating the cells with 0.05% Trypsin-EDTA (#25300-062, Gibco Life technologies™) for 10 minutes at 37°C. Cells from two DG were then cultured in 5ml of serum-free Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 + GlutaMAX™ (#31331-028, Life technologies™) supplemented with 10ng/ml of epidermal growth factor (EGF, #53003-018, Life technologies™), 5ng/ml of recombinant human basic fibroblast growth factor-2 (bFGF-2, #13256-029, Life technologies™), 100U/ml Penicillin and 100μg/ml Streptomycin (PenStrep, #15140-122, Life technologies™), and 0.5X of B27 Supplement (#17504-044, Life technologies™). Cells were grown at 37°C in a 95% air and 5% CO_2 humidified atmosphere. Seven days after the isolation of DG cells, the resulting neurospheres were seeded in multiwells coated with 0.1mg/ml poly-D-lysine in serum free DMEM/F-12, GutaMAX medium supplemented with PenStrep and B27 supplement, devoid of growth factors to promote cell differentiation. Neurospheres were plated into glass coverslip (Ø=10mm) in 24 multi-well plates for immunocytochemistry studies and in 6 multi-well plates for mRNA or protein extraction.

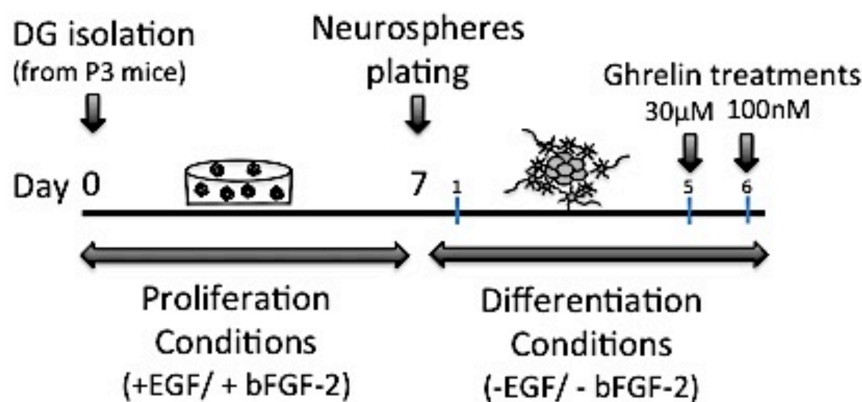


Figure 2.1: Schematic representation of the neurospheres cell culture protocol. DG, dentate gyrus; P3, postnatal 3-days old mice; EGF, epidermal growth factor; bFGF-2, basic fibroblast growth factor-2.

2.2 RNA isolation from neural progenitor cells

For the characterization of the temporal pattern of GHSR1 mRNA expression during neural progenitor cells (NPCs) differentiation, neurospheres cell culture were collected before (day 0) and 1, 3, 5, 7 and 10 days after plating. We used hypothalamic tissue as our positive control.

RNA extraction was performed using the Aurum™ Total RNA Mini Kit (#732-6820, Life Science Research, BIO-RAD), following manufacturer instructions. Cells were lysed with the provided lysis buffer supplemented with 1% β-mercaptoethanol (#63689, Sigma-Aldrich) and lysate collected by thoroughly scraping the wells. The lysate was then frozen and stored at -20°C until further use. After defreezing, 70% ethanol was added to each sample tube, followed by vortexing and up-and-down pipetting to ensure proper mixing. The homogenized lysates were transferred into RNA binding columns and centrifuged for 30 seconds at 20827x*g* (the same speed was used for all centrifugations in this protocol). Columns were rinsed with a low stringency wash solution, centrifuged for 30

seconds and treated with 5µl of DNAse I (reconstituted with 250µl 10nM Tris, pH 7.5) diluted in 75µl of DNAse dilution solution (provided with the kit) for 15 min, at room temperature in the dark. Then, columns were rinsed with a high stringency wash solution, centrifuged for 30 seconds, rinsed again with low stringency wash solution, and then centrifuged for 1 minute and 2 extra minutes. Purified RNA was collected by filling the columns with the provided elution solution and 2 minutes of centrifugation. RNA was quantified by measuring optical density (OD) at 260nm and purity analyzed using the ratio OD_{260}/OD_{280}, with NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.3 Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR)

Extracted RNA (≅500ng) was reverse transcribed on a UnoCycler (VWR International) using iScript™ cDNA synthesis kit (#170-8890, Bio-Rad) and following manufacturer's instructions. A reaction mix containing iScript™ reverse transcriptase, RNAse H^+, oligo-dTs and random hexamers and nuclease-free water was added to the extracted RNA, making a final volume of 20µl. A negative control was used for qPCR by submitting samples to the mix devoid of the reverse transcriptase (RT- samples). The complete reaction mixes were incubated during 5min at 25°C for annealing, followed by an extension step at 42°C for 30min, and 5min at 85°C for melting and RNA degradation. The generated cDNA was stored at -20°C until use.

The qPCR of the cDNA products was performed on CFX96 Touch™ Real-Time PCR Detection System/ C1000 Touch™ Thermal Cycler (Bio-Rad), using iQ™ SYBR® Green supermix reaction kit (#170-8882, Bio-Rad). Amplification was done in a solution containing 2.5µl of the cDNA template (1:10), 5µl of 2xiQ™ SYBR Green® supermix (containing iTaq™ DNA polymerase, deoxynucleotide mix, $MgCl_2$, SYBR Green® I, enhancers and stabilizers), forward (F) and reverse (R) primers (Table 2.1) and nuclease-free water to complete 10µl of reaction volume. To detect contaminations and/or amplification of genomic DNA RT- samples were used as negative controls. All reactions were performed in duplicate. Three different housekeeping genes were tested: *Hprt1, Hypoxanthine phosphoribosyltransferase 1; β-actin* and *Gapdh, glyceraldehyde-3-phosphate dehydrogenase*. *Gapdh* mRNA expression was demonstrated the most stable in our cultures. Thus, we used *Gapdh* cDNA detection as reference for our qPCR analysis.

Two primers were used for the detection of GHSR1: a pair to detect the Ghsr1a targeting the mouse Ghsr mRNA (exon 3), and another one targeting the exon 2 of the mouse Ghsr1 mRNA. The primers used to recognize Ghsr1 exon 2 were designed using the open-source software tool PerlPrimer. The other primers were purchased from Sigma-Aldrich.

qPCR protocol consisted in two phases, an initial phase of denaturation and enzyme activation step at 95°C, followed by 40 cycles of denaturation (95°C), and annealing (60°C) (elongation occurred during the temperature ramp between annealing and denaturation). Fluorescence was determined at the end of the annealing step and melting curves were calculated for all samples between 55 and 95°C, with a temperature increase of 0.5°C every 10seconds and continuous fluorescence measurement.

Gene	Concentration	Primer Sequence (5'-3')
βIII-*Tubulin*	0.3µM	**F-**CAACTATGTAGGGGACTCAG **R-**CCACTCTGACCAAAGATAAAG
Doublecortin	0.3µM	**F-**GGATGAGAATGAATGCAGAG **R-**CTTTGACTTAGGTGTTGAGAG
Gapdh	0.3µM	**F-**TGGCCTTCCGTGTTCCTAC **R-**GAGTTGCTGTTGAAGTCGCA
Ghrelin	0.3µM	**F-**AGATCAGGTTCAATGCTCC **R-**ATCCTGAAGAAACTTCCCC
Ghsr1a	0.5µM	**F-**ACAAACAGACAGTGAAGATG **R-**TGTAGAGAATGGGGTTGATG
Ghsr1 Exon 2	0.3µM	**F-**TACTTCGCCATCTGCTTCC **R-**ATCTGTGCCGTTCTCGTG

Table 2.1: Primer sequences (F and R) and concentrations used for RT-qPCR analysis.

2.4 qPCR analysis

Real-time qPCR results were analyzed using LinRegPCR, a program for the analysis of quantitative RT-PCR data. This software uses an algorithm to analyze raw data (non-baseline corrected data) and determine the optimal baseline, the mean PCR efficiency per amplicon and the Cq value per sample based on the log-linear phase of the amplification reactions (Ramakers et al. 2003; Ruijter et al. 2009). Cq represents the fractional number of cycles needed to reach a determined fluorescence threshold at the exponential phase of the fluorescent curve. The relationship between mRNA expression in control and experimental samples was analyzed using the following equation:

$$\text{Ratio} = \frac{\text{Mean}\left[\dfrac{E_{target}^{\;Cq_c}}{E_{reference}^{\;Cq_c}}\right]}{\text{Mean}\left[\dfrac{E_{target}^{\;Cq_s}}{E_{reference}^{\;Cq_s}}\right]}$$

where **'**E' refers to the efficiency of the qPCR reactions for the selected amplicon/primer pair; 'Cq_C' is the Cq of the control sample (day 0 after plating) and 'Cq_S' the Cq of the samples in study (day 1, 3, 5, 7 and 10). 'Target' refers the to mRNA of interest that is normalized by the 'reference' gene mRNA (*Gapdh*).

2.5 Western blotting

NPCs were collected by scraping in a cold-lysis buffer containing 150mM of NaCl (#7647-14-5, Fisher Scientific), 50mM of Tris Base pH 8.0 (#T1378, Sigma-Aldrich), 1mM EGTA (#E4378, Sigma-Aldrich), 5mM EDTA (#ED2p, Sigma-Aldrich), 1% Triton X-100 (#BP151-500 Thermo Fisher Scientific), 0.1% sodium dodecyl sulfate (SDS, #05030, Sigma-Aldrich), 1mM of phenylmethylsulfonyl fluoride (PMSF, #P7626, Sigma-Aldrich) and protease Inhibitor Cocktail (#P8340, Sigma-Aldrich). Samples were stored at -20 °C until further use. Hippocampal and hypothalamic tissues were used as positive controls. Cells/ tissues were sonicated and centrifuged at 20817x*g* for 10 min at 4°C. The pellet was discarded, the supernatant collected and total protein was quantified using the Bradford assay (#500-0006, BIO-RAD). Bovine serum albumin (BSA, #9048-46-8, Santa Cruz Biotechnology) was used as protein standard for the calibration curve. Samples were denatured using sodium dodecyl sulfate (SDS) sample buffer 6X composed of 0.5MTris-HCL pH 6.8, 30% glycerol (#24387.361, VWR PROLABO), 10% SDS, 0.6M dithiothreitol (DTT, #BP172-25, Fisher Scientific), 0.012% bromophenol blue sodium salt (#34725-61-6, Sigma), and boiled at 95°C for 5min. 30µg of denatured protein per sample were loaded onto 4% stacking/10% resolving acrylamide/bisacrilamide gel (#BP14101, Thermo Fisher Scientific) and separated by SDS-PAGE using a 1M Bicine (#B3876, Sigma-Aldrich)/ 0.5% SDS electrophoresis buffer pH 8.3, at constant voltage (120 V) at room temperature. After separation, proteins were transferred to a Polyvinylidene difluoride (PVDF) methanol-activated membrane (#162-0264, BIO-RAD) under constant amperage (75 mA), for 2 hours at 4°C in a solution containing 10mM CAPS (#C6070, Sigma-Aldrich) and 10% methanol (VWR International) at pH 11. Membranes were blocked in Tris-buffer saline (TBS, 15mM Tris, 150mM NaCl, pH 7.6) containing 5% w/v BSA and 1% v/v Tween® 20 (#P5927, Sigma-Aldrich) for 1 hour, at room temperature, followed by an overnight incubation at 4°C with primary antibody solution (table 2.2) diluted in 5% BSA. Some membranes were also incubated with mouse anti α-tubulin (1:1000) (T-6199, Sigma). After rinsing three times for 10 min with TBS-T, membranes were incubated for 1 hour at room temperature with the corresponding alkaline phosphatase-linked goat anti-rabbit IgG or anti-mouse IgG (1:20000), in TBS-T with 5% BSA. Protein immunoreactive bands were visualized in a Versa-Doc 3000 Imaging System (Bio-Rad) after incubation of membranes with ECF chemifluorescent reagent (#RPN5785, GE Healthcare Life Sciences).

GHSR1 Antibodies			
Company	**Reference**	**Recognition site**	**Dilution**
Santa Cruz	Sc-10362	N-term	1:500
Abcam[1]	Ab95250	Middle (150-250)	1:1000
Phoenix	H-001-62	C-term (330-366)	1:1000
Abcam[2]	Ab125457	C-term (316-365)	1:1000

Table 2.2: List of primary antibodies used to characterize the GHSR1 by Western Blotting.

2.6 Enzyme–Linked Immunosorbent Assay (ELISA)

Neurospheres conditioned media from days 5 and 6 after plating were collected and ghrelin content analyzed with an ELISA Kit (RayBiotech, #EIAM-GHR). The ELISA kit is supplied with a 96-microplate pre-coated with anti-rabbit secondary antibody and all the necessary reagents: lyophilized anti-ghrelin polyclonal antibody, 1X assay diluent E (diluent for both standards and samples), lyophilized biotinylated ghrelin peptide, lyophilized standard ghrelin peptide, lyophilized positive control, wash buffer concentrate (20x), horseradish peroxidade (HRP)-streptavidin concentrate, 3,3',5,5'-tetramethylbenzidine (TMB) one-step substrate reagent and a stop solution. A blocking step and incubation of the plate with anti-ghrelin antibody was done overnight at 4°C. Then, the solution was discarded and plate rinsed 4 times with 1x wash buffer, followed by 2.5 hours incubation with biotinylated ghrelin peptide and peptide standard in samples at room temperature. The solution was discarded and plate rinsed again 4 times, and then incubated for 45 minutes with HRP-streptavidin solution. Next, a stop solution was added after incubation with TMB substrate reagent in the dark, and the absorbance was immediately read at 450nm in a spectrophotometer (SPECTRAmax Plus® 384, Molecular Devices Corporation). Three positive controls were used: a cell culture medium sample (supplied with the ELISA kit), and cell culture media of neurospheres treated with two concentrations of ghrelin (30µM and 100nM).

2.7 Ghrelin Treatments

Rat ghrelin (AnaSpec, #AS-24160) stock solutions were prepared in sterile phosphate-buffered saline pH 7.4 (PBS, containing 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4) and stored at -20°C until use. Neurosphere cultures under differentiation conditions were treated with vehicle (sterile PBS) or ghrelin at two different final concentrations: 30µM at day 5 and 100nM at day 6 after plating, for 24h and 8h, respectively. After treatments, cells were washed with PBS 3 times, fixed with 4% Paraformaldehyde prepared in 0.2M phosphate buffer (PFA, #P6148, Sigma-Aldrich) for 15minutes, rinsed again with PBS (3 times, 5 min each) and stored in PBS at 4°C until further use. Concentrations for ghrelin treatments were chosen based on data from previous published studies (Johansson et al. 2008; Chung et al. 2013).

2.8 Immunofluorescence

Cells were washed for 5 minutes with PBS 3 times, fixed with 4% PFA for 15 minutes, rinsed again with PBS and incubated for 1h at room temperature with a blocking solution containing 3% BSA and 0.5% Triton X-100 diluted in PBS. Incubation with primary antibodies (table 2.4) diluted in 3% BSA, 0.2% Triton X-100 and PBS was performed overnight at 4°C in a humidified chamber. Cells were washed with PBS and incubated for 2 hours at room temperature with the corresponding secondary antibodies diluted in 3% BSA, 0.2% Triton X-100, and Hoechst 33342 (2µg/ml, Molecular Probes, Life Technologies). Finally, coverslips were mounted in Fluoroshield mounting medium (#ab104135, Abcam). Controls without primary antibodies were performed for each experiment, to exclude non-specific labeling.

Primary Antibodies	Dilution	Reference
Goat α- DCX	1:500	Santa Cruz, sc-8066
Goat α-GFAP	1:4000	Abcam, ab53554
Rabbit α-GHSR1	1:500	Abcam, ab95250
Rabbit α-Ki67	1:2000	Abcam, ab16667
Mouse α-MAP2	1:500	Abcam, ab11267
Mouse α-Nestin	1:500	BD Pharmingen, 556309
Mouse α-NeuN	1:4000	Millipore, MAB377
Goat α-Sox2	1:500	Santa Cruz, sc-17320
Secondary Antibodies	**Dilution**	**Reference**
Donkey anti- Goat 488	1:500	Life technologies, #A11055
Donkey anti- rabbit 594	1:500	Life technologies, #A21207
Donkey anti- mouse 647	1:500	Life technologies, #A21235

Table 2.4: list of primary and secondary antibodies used for immunofluorescence. DCX, doublecortin; GFAP, glial fibrilary acidic protein; GHSR1, growth hormone secretagogue receptor 1; NeuN, neuronal nuclear antigen (FOX-3); Sox2, SRY (sex determining region Y)-box 2.

2.9 Image analysis of ghrelin treated neurosphere cultures

In order to evaluate the effect of ghrelin treatments, neurosphere cultures were immunostained to detect the cell proliferation marker Ki67, the NPCs marker nestin, the neuronal marker doublecortin and nuclei counterstained with Hoechst. Images from immunofluorescent stainings were acquired using a widefield fluorescence microscope (Observer, Carl Zeiss, Göttingen, Germany). Each experimental condition was performed in triplicate. 5 images were taken from the center of 5 randomly selected neurospheres (Fig. 2.2A) and 20 images from their periphery (carpet regions, Fig. 2.2B), using a 20x magnification objective. Images were analyzed with NIH ImageJ Software (Schneider *et al.* 2012). Three ImageJ macros were generated to help on cell quantification. Regions of Interest (ROI) were manually defined to select the neurosphere center (Fig.2.2A) or exclude, when analyzing the carpet (Fig.2.2B). Then, two different pixel intensity thresholds were manually selected in Hoechst channel to further segment pyknotic bodies (highly and homogeneously stained round nuclei, Fig.2.2C) and alive cells (bigger nuclei showing weak staining and highly stained clusters of heterochromatine, Fig.2.2D). A macro was designed to help on the selection of pixel intensity thresholds to detect Ki67, DCX and Nestin positive cells. Briefly, user manually counts each population of cells in several images. Then, an algorithm is applied to get the threshold that allows the detection of a similar number of cells and storage this information in a table. The final macro automatically analyzes the percentages of different populations of cells by using previous established intensity thresholds to eliminate pyknotic bodies, segment nuclei and analyze staining.

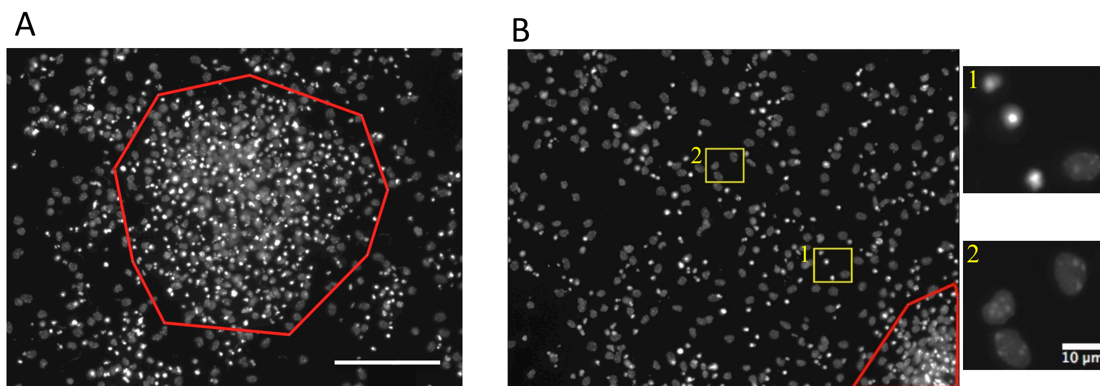


Figure 2.2: Representative images of neurosphere center/neurosphere carpet stained with Hoechst. (A) Image of the center of a neurosphere at 6 days after plating, showing the area considered for quantification (Region of Interest, ROI) delimited with a red line. (B) Image of the periphery (cells carpet) of a neurosphere, red line delimits a region that was excluded in the analysis as it belongs to the center of a neurosphere. Scale bar: 100µm. Magnification of (1) pyknotic bodies and (2) alive cells of the highlighted yellow boxes on (B). Scale bar: 10µm.

2.10 Analysis of cells expressing the GHSR1

To analyze the type of cells that express GHSR1, 25 images were obtained under a 40x objective and analyzed with Cell Profiler (Carpenter, Anne E *et al.* 2006). Cell profiler is a cell image analysis software that allows the user to create modules that can be placed in sequential order to form a pipeline; the pipeline is used to identify and measure biological objects and features in images obtained by fluorescence microscopy. For the analysis, subtract background function from ImageJ (Schneider *et al.* 2012) was applied for each channel using a 200micrometer rolling ball radious. Then, a module identified nucleus and cells, based on size and intensity of the objects. The intensity and shape of the objects in the Hoechst channel are measured, and bigger nuclei showing weak staining and highly stained clusters of heterochromatine were considered alive cells, while smaller and highly homogeneous stained round nuclei were considered pyknotic cells (Fig. 2.2). Once alive cells were segmented, another filter was applied to identify each type of cell and presence of GHSR1 based on the intensity of the staining in each channel. Data were exported to an excel file table, and the percentage of different population of cells and cells expressing GHSR1 was calculated.

2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, California, USA). Data are presented as mean ± standard error of the mean. Statistical significance was determined using one way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test for qPCR data. For the analysis of ghrelin treatments effect, Student's t test were used when data were parametric, or Wilcoxon matched pairs was used when data were non parametric. Statistical significance was considered relevant for p values <0.05.

Chapter 3

Results

3.1 Ghsr1 mRNA expression on DG neurosphere cell cultures

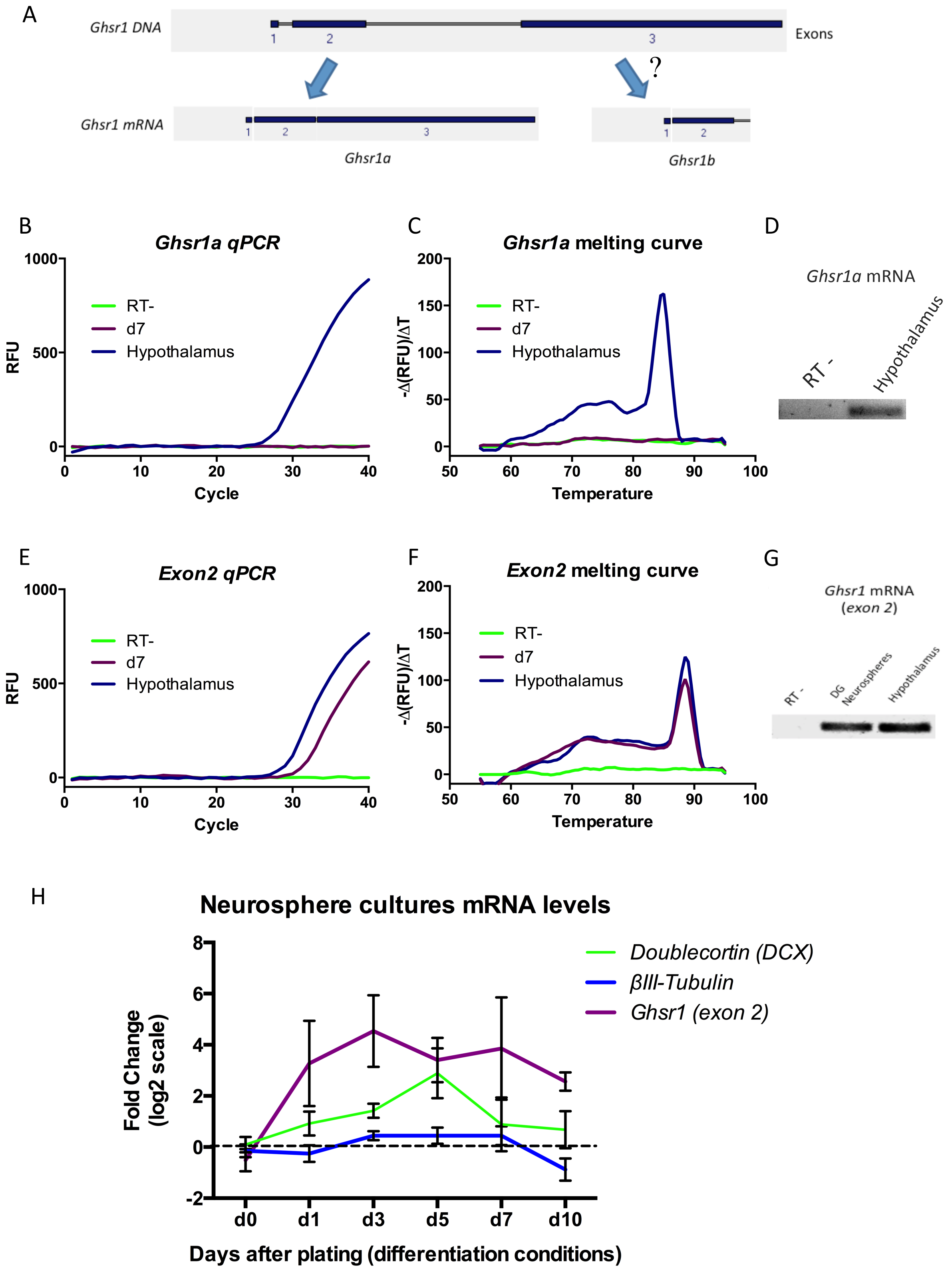
In order to study the relevance of GHSR-1a in the modulation of DG NPCs derived cell cultures, we evaluated *Ghsr1* mRNA expression at different time points after neurosphere plating. First, we analyzed the structure of the mouse *Ghsr1* gene using the mouse genomic DNA sequence (http://www.ncbi.nlm.nih.gov/gene/208188) and the *Ghsr-1a* mRNA sequence (http://www.ncbi.nlm.nih.gov/nuccore/146198834/). The comparison of these two sequences, using the software PerlPrimer, indicates that mouse *Ghsr1* gene contains three exons (Fig. 3.1A). This result differes from what has been described for rat and human genes that present two exons (Howard *et al.* 1996). Therefore, to analyze the expression of the *Ghsr-1a* mRNA we used a commercial pair of primers targeting exons 2 and 3. Using these primers we were unable to detect mRNA levels of *Ghsr-1a* in our DG neurosphere cultures before (day 0) or after plating, while we observed *GHSR-1a* mRNA expression in hypothalamic cDNA used as positive control (Fig. 3.1B).

By comparison with human and rat data about *Ghsr1* gene, we supposed that mouse *Ghsr1* gene could present two splicing variants (Fig. 3.1A). Therefore, we designed another pair of primers targeting mouse *Ghsr1 exon 2*. These primers allow the detection of any mRNA splice variants containing exon *2*, e.g. *Ghsr-1a* and the predicted *Ghsr-1b* mRNAs. When using these primers raised against exon *2*, we did observe *Ghsr1* cDNA amplification on our DG neurosphere cultures and in hypothalamus samples (Fig. 3.1E). The specificity of the amplification reaction was verified using negative controls (RT-), analyzing melting curves (Fig 3.1C and Fig. 3.1F) and checking the size of the amplification product (Fig. 3.1G and Fig. 3.1D). These data suggest the presence of the *Ghsr-1b* transcript in our cultures.

We further analyzed the temporal expression of *Ghsr1* mRNA using the pair of primers that targets the *exon 2* of *Ghsr1*. We observed that *Ghsr1* mRNA levels increase early after plating, showing a trend for increased mRNA expression from day 1. However, no significant differences between time points were detected. We also observed doublecortin (*DCX*, a marker for neuroblasts/young neurons) mRNA expression in our cultures, showing a peak at day 5 post-plating. Our cultures show detectable levels of *βIII-tubulin*, which remains unchanged along the time.

This study served to select the post-plating date for further analysis, days 5-6 post-plating, since it represents the days when *Ghsr1* mRNA expression were increased, and as an advantage, it doesn't require too much cell culturing time.

Our data indicates that in DG neurosphere cultures there is no expression of *Ghsr-1a* mRNA, while another *Ghsr1* splice variant containing exon 2 is present in these cultures.



A
Ghsr1 DNA
Exons
?
Ghsr1 mRNA
Ghsr1a
Ghsr1b
B
Ghsr1a qPCR
RFU
Cycle
RT-
d7
Hypothalamus
C
Ghsr1a melting curve
-Δ(RFU)/ΔT
Temperature
D
Ghsr1a mRNA
RT -
Hypothalamus
E
Exon2 qPCR
F
Exon2 melting curve
G
Ghsr1 mRNA (exon 2)
H
Neurosphere cultures mRNA levels
Fold Change (log2 scale)
Doublecortin (DCX)
βIII-Tubulin
Ghsr1 (exon 2)
Days after plating (differentiation conditions)

Figure 3.1: Analysis of Ghsr1 mRNA expression on DG neurosphere cultures. (A) *Ghsr1* gene consists in 3 exons as indicated by the analysis of its genomic and mRNA sequences. By comparing human and rat data about *Ghsr1* gene, we inferred that mouse *Ghsr1* gene may present two splice variant, GHSR-1a and GHSR-1b. (B) Relative fluorescence (RFU) and (C) melting curve charts showed no amplification of *Ghsr-1a* cDNA from DG neurospheres (purple). In hypothalamic tissue *Ghsr-1a* cDNA was amplified (blue). Image from an agarose gel showing specific bands corresponding to the expected size of (D) Ghsr1a and (G) Ghsr1 amplified products. (E) RFU and (F) melting curve charts showed that Ghsr1 cDNA is amplified in our neurosphere samples when using primers targeting *exon 2*. (H) Levels of *Ghsr1* mRNA (purple) show a trend to increase early after plating. DG neurosphere cultures presented detectable levels of *Dcx* mRNA (green) and βIII-tubulin mRNA (blue). N=3-5.

3.2 GHSR1 protein detection

In order to detect GHSR1, we tested four commercially available antibodies (some of them previously used to describe GHSR1 presence in cells of the neurogenic niches) directed against different regions of GHSR1 (Fig. 3.2A). We used western blotting of protein extracts from hypothalamus and hippocampus, CNS regions that have been previously shown to present high levels of GHSR1. Santa Cruz (#sc-10362) (Fig. 3.2B) and Phoenix antibodies (#H-001-62)(Fig. 3.2D) that target the N-terminal region of GHSR1 and Abcam[2] antibody (#ab125457)(Fig. 3.2E) that targets the C-terminal region of GHSR1 presented many bands. However, the antibody from Abcam[1] (#ab95250)(Fig. 3.2C), that targets the middle region of GHSR1 shows two bands around 42 and 32kDa. Importantly, 42 and 32 kDa are the molecular sizes expected for GHSR-1a and GHSR-1b, respectively (Leung, Po-Ki et al. 2007). Based on these results, we decided to use the antibody from Abcam[1] (#ab95250) for further analysis.

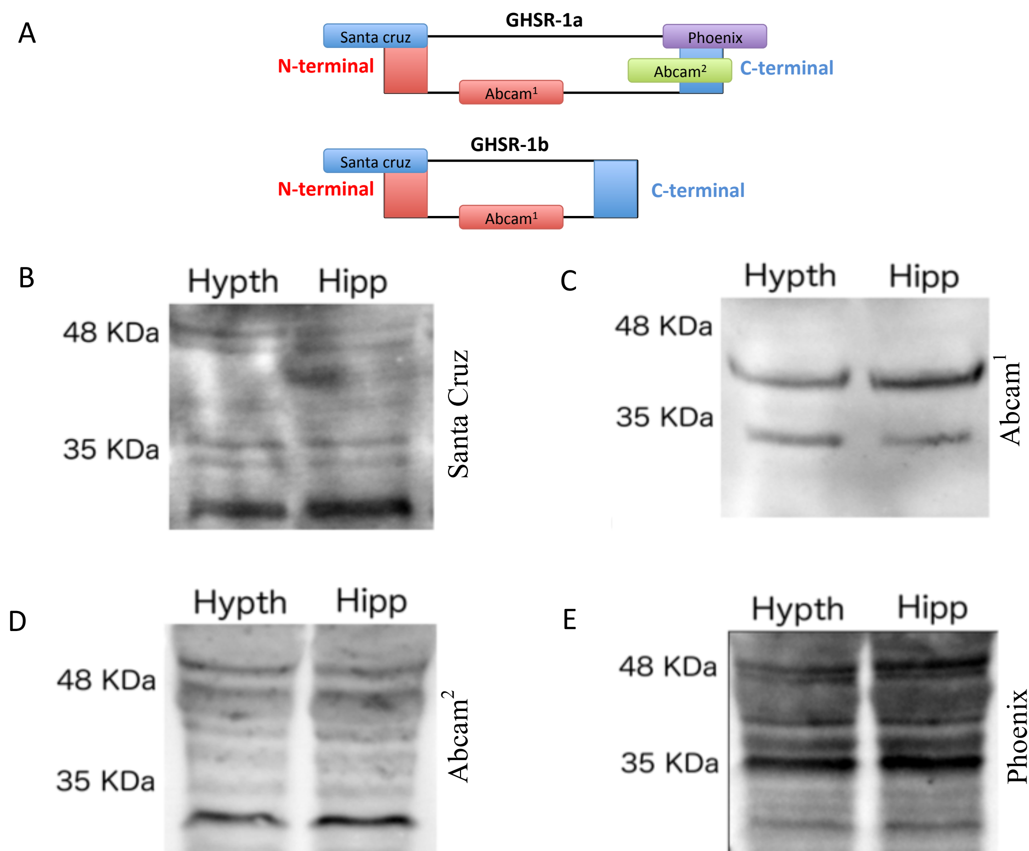


Figure 3.2: Characterization of different GHSR1 antibodies. (A) Several GHSR1 antibodies were tested by western blotting using 30µg of cell extracts from the hypothalamus and the hippocampus. Different band patterns are observed: the antibodies from (B) Santa Cruz, (D) Abcam[2] and (E) Phoenix exhibit many several bands, while (C) Abcam[1] presented only two bands at 42 and 32kDa. Hipp, hippocampus; Hypth, hypothalamus.

We evaluated GHSR1 protein expression on neurospheres 5 days after plating. Hippocampus and DG tissue samples were used as positive controls. Our western blotting membranes showed a band with 42 kDa (the predicted size for GHSR-1a) in the hippocampus and DG samples. However, the samples from our neurosphere cultures showed a new band with an unexpected size (between 42 and 32kDa). A band around 32 kDa was also observed (Fig. 3.3A). We also detected the presence of GHSR1 in our cultures using immunofluorescence staining. In agreement with qPCR data, we observed increased levels of GHSR1 and doublecortin at day 5 after plating when compared to day 1 (Fig. 3.3B).

Our data indicates that the antibody from Abcam[1] (#ab95250) specifically recognizes GHSR1, and suggests that its truncated isoform, GHSR-1b, is present in mouse tissue and DG neurosphere cell cultures.

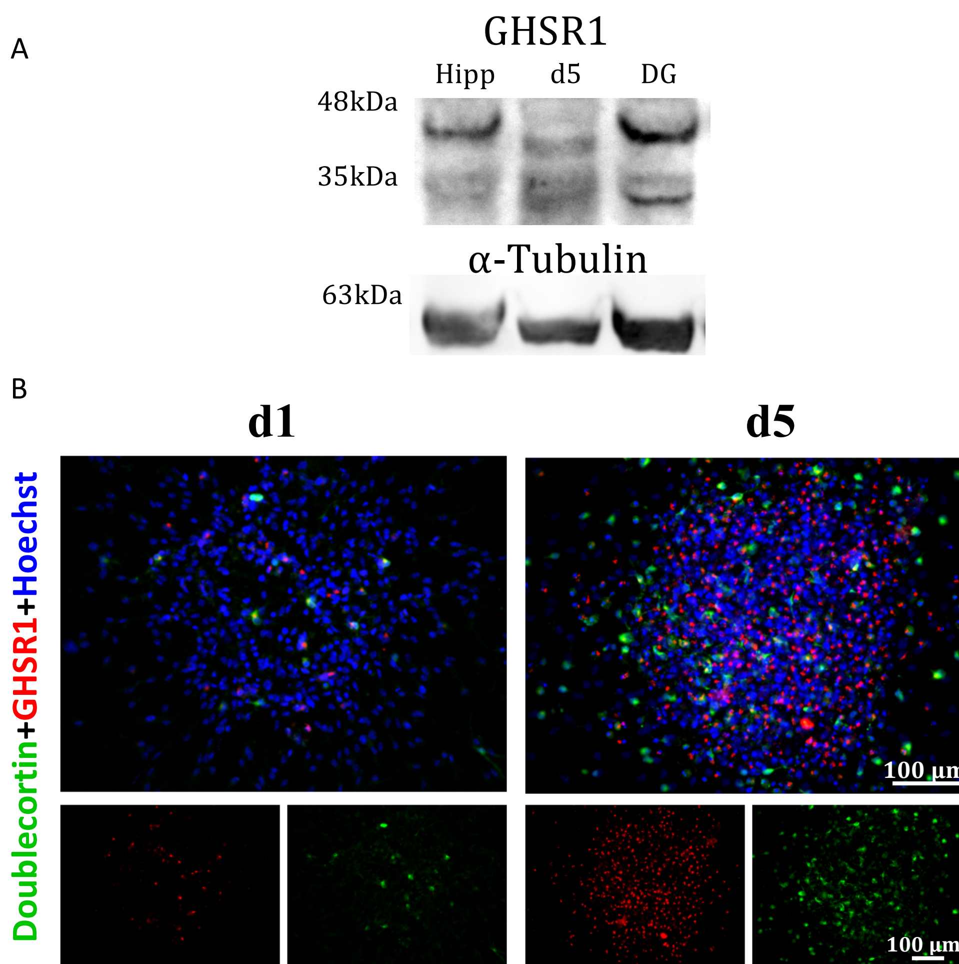


Figure 3.3: GHSR1 detection in DG neurosphere cultures. (A) Western blotting of 30µg cell extracts from hippocampus, neurosphere at day 5 after plating and DG samples. Hippocampus and DG samples showed two bands around 42 and 32kDa. D5 neurosphere sample presented an unexpected band between 42 and 32kDa, and another band around 32kDa. (B) Immunofluorescence images showing GHSR1 (red) and DCX (green) in DG neurospheres at day 1 and day 5 after plating. An increase in DCX and GHSR1 immunofluorescence staining is observed in d5 neurosphere cultures when compared to d1 cultures. Cell nuclei were stained with Hoechst 33342 (blue). DG, dentate gyrus; d1, day 1 after plating; d5, day 5 after plating; GHSR1, growth hormone secretagogue receptor 1; Hipp, hippocampus.

3.3 GHSR1 expression in NPCs and differentiating neurons

Previous studies suggest that GHSR1 is expressed in the hippocampus (Diano et al. 2006). However, it has not been fully described the subcellular localization of GHSR1 in hippocampal NPCs and derived cells. Here, we first analyzed the subcellular pattern of expression of GHSR1 in our neurosphere cell cultures and used cortical neurons (kindly given from Dr. Ana Luísa Carvalho laboratory) as a system where GHSR-1a has been previously described (Ribeiro et al. 2014).

Cortical neurons maintained 15 days *in vitro* GHSR1 sataining in their soma (perinuclear) and throughout cell dendrites (stained with microtubule associate protein, MAP2). However, DG neurosphefere derived cells did not express the same staining pattern as cortical neurons, exhibiting only a perinuclear staining (Fig. 3.4). Importantly, GHSR-1a has been described to be located in neuronal dendrites (Ribeiro et al. 2014), while GHSR-1b is mainly present in the ER, showing a perinuclear pattern (Leung et al. 2007; Chow et al. 2012). The presence of a perinuclear pattern of staining and the absence of a punctated and dendritic one in our cultures, suggest that only GHSR-1b is expressed by our cells. This is in agreement with our previous qPCR data.

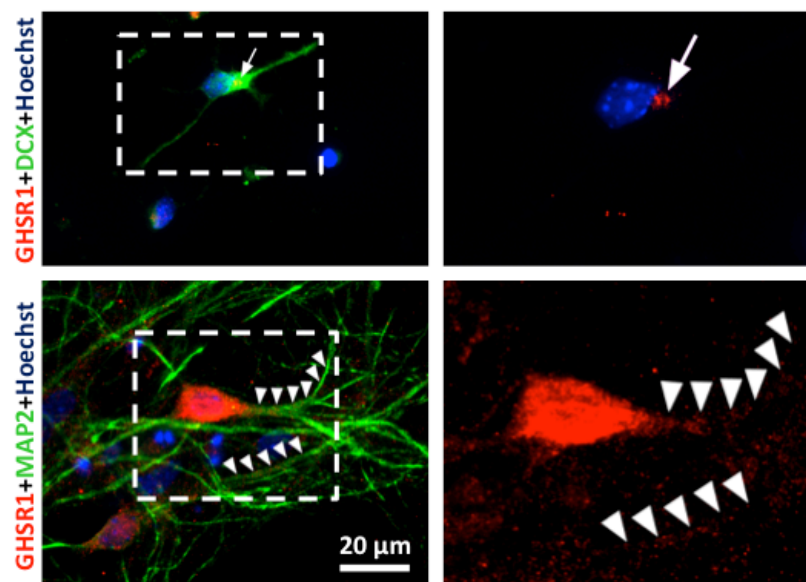


Figure 3.4: Immunofluorescence staining pattern for GHSR1. Immunofluorescence images of d6 neurosphere cultures showing a DCX+ (green) cell expressing GHSR1 (red) with a specific perinuclear-staining pattern (top panels arrow). 15-DIV cortical neurons exhibit two different staining patterns for GHSR1: a punctated and dendritic pattern that co-localizes with MAP2 (green) and a perinuclear one (lower panels arrow heads). DCX, doublecortin; DIV, days in vitro; MAP2, microtubule-associated protein 2; GHSR1, growth hormone secretagogue receptor 1. Scale bar of lower panels applies to top panels.

Our previous results suggest that a truncated isoform of GHSR1 is expressed in DG neurosphere cultures. To explore whether this expression could be relevant, we analyzed the type and proportion

of cells showing immunofluorescence staining for ghrelin receptor. We observed that some NPCs (Nestin+/Sox2+, Fig. 3.5A) and many neuronal cells (DCX+, NeuN+ and DCX+/NeuN+, Fig. 3.5B) were stained for GHSR1 at the perinuclear region. However, astrocytes (cells showing high levels of GFAP) did not expressed GHSR1 (Fig. 3.5C).

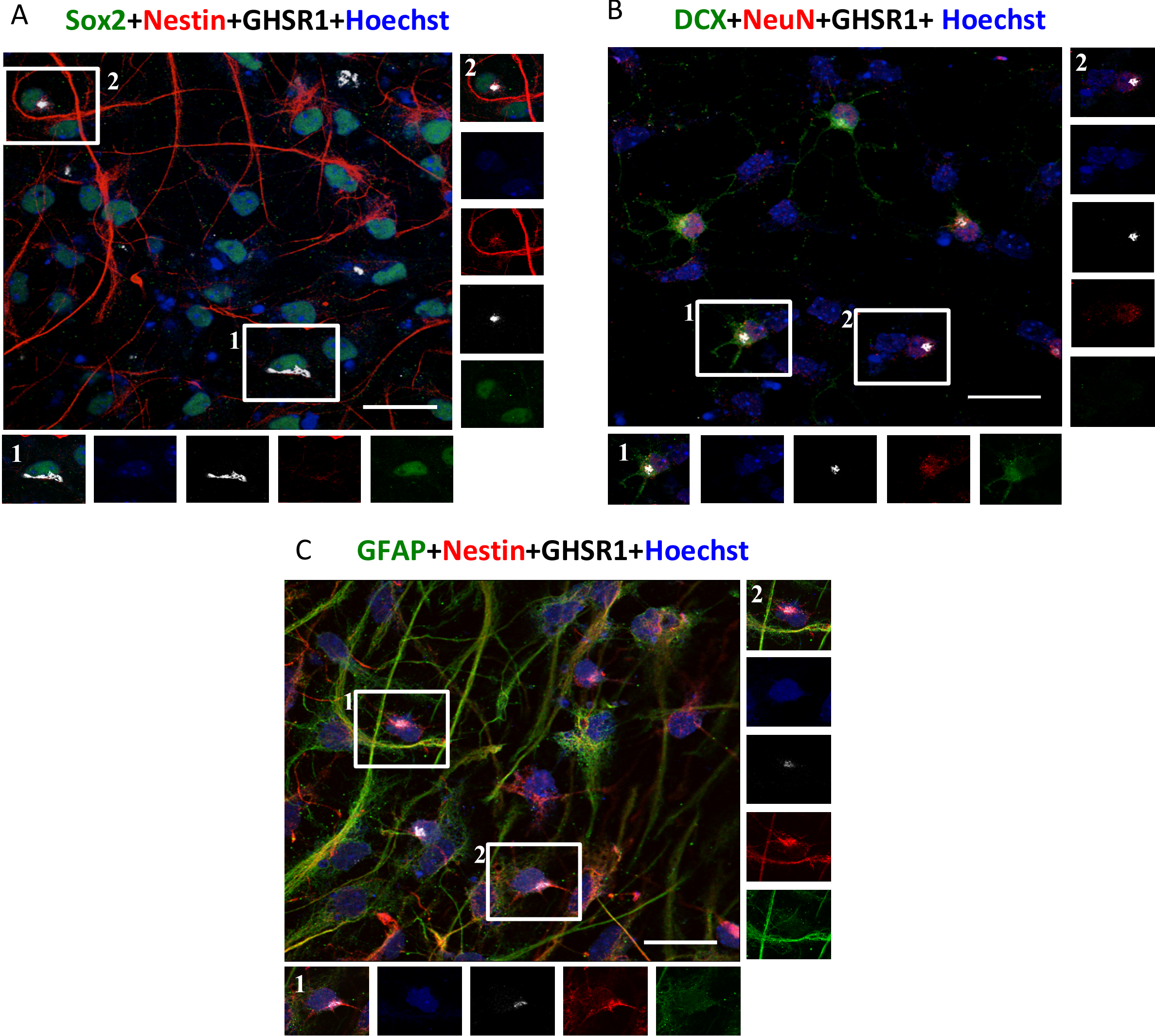


Figure 3.5: GHSR1 expression in NPCs and NPCs derived cells. (A) Immunofluorescence image showing Sox2+ (green), Nestin+ (red) and GHSR1+ (white) stained NPCs. (B) Fluorescence image showing DCX (green) and NeuN (red) positive cells expressing the GHSR1 (white). (C) Immunofluorescence staining for GFAP (green), Nestin (red) and GHSR1 (white). DCX, doublecortin; GFAP, glial fibrilary acidic protein; GHSR1, growth hormone secretagogue receptor 1; NeuN, neuronal nuclear antigen (FOX-3); Sox2, sex determining region Y (SRY)-box 2. Scale bars: 20µm

As many neuronal cells expressed the receptor, we decided to further explore the proportion of these cells in our cultures. It is important to point out that the majority of cells of our cultures (82 ± 2.7 %) did not belong to the neuronal lineage (NeuN-/DCX-)(Fig. 3.6A). Remarkably, our cell cultures showed a 17 ± 2.3% of neuronal cells (Fig. 3.6A). From this percentage of neuronal cells, only 1.4 ± 0.1% corresponded to the less mature neuronal stage (DCX+/NeuN-), while 7.8 ± 2% were neurons in

an intermediate phase (NeuN+/DCX+) and 8.8 ± 2% were mature neurons (NeuN+/DCX) (Fig. 3.6A). Regarding the expression of GHSR1, 15.87 ± 2% of cells in our cultures expressed the receptor and 59 ± 10% of these cells were from the neuronal lineage. Within the population of neuronal cells, 50 ± 9% of the immature neurons (DCX+/NeuN-), 62 ± 9% of NeuN+/DCX+ cells and 44 ± 4% of the mature neurons (NeuN+/DCX-) expressed the receptor (Fig. 3.6B). By contrast, only 8 ± 2% of non neuronal cells (NeuN-/DCX-) were stained for GHSR1 (Fig. 3.6B).
Taken together, our results indicate that the expression of GHSR1 is highly relevant for neuronal cells in DG neurosphere cultures, since more than half of these cells showed high levels of perinuclear receptor staining.

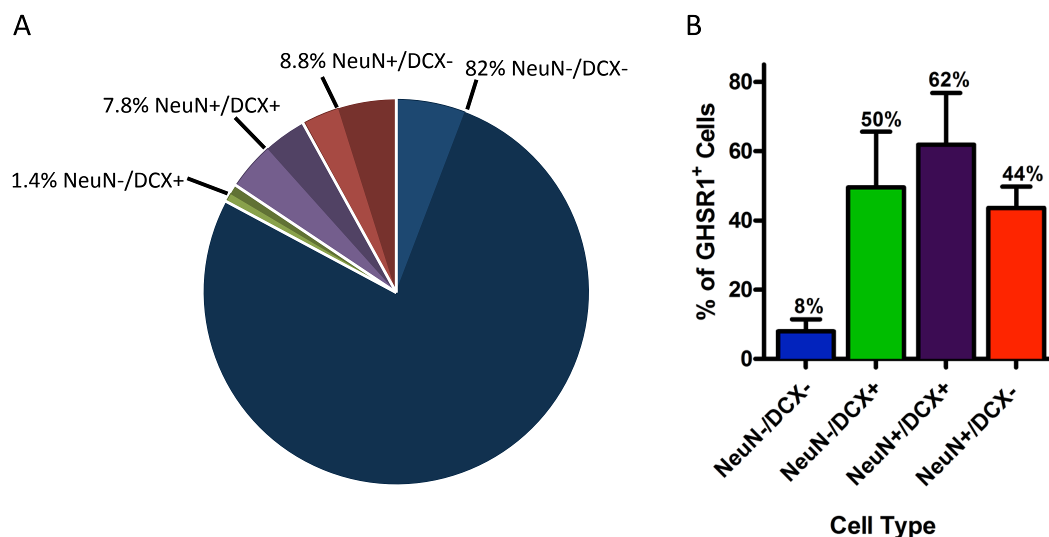


Figure 3.6: Quantification of cells expressing GHSR1. (A) Pie- chart showing the proportion of cells present in our DG neurosphere cultures that are not from the neuronal lineage (blue), immature neurons (green), maturating neurons (purple) and mature neurons (red). Light colors represent the percentage of cells expressing GHSR1 in each population. (B) Graph representing the type and percentage of cells expressing GHSR1. N=3. DCX, doublecortin; GHSR1, growth hormone secretagogue receptor 1; NeuN, neuronal nuclear antigen (FOX-3).

3.4 Ghrelin detection in DG neurosphere cell cultures

Despite ghrelin is mainly produced in the stomach, there are several evidences indicating that it can be synthesized locally in the CNS. Thus, we decided to explore whether our cell cultures could be producing ghrelin. Surprisingly, we were able to detect *ghrelin* mRNA in our DG neurosphere cultures by qPCR (Fig. 3.7A). The specificity of the amplification reaction was verified by analyzing the melting curve (Fig. 3.7B) and the size of the amplification product in a positive control sample from hypothalamus (Fig. 3.7C). We also analyzed *ghrelin* mRNA expression along different time points and observed that *ghrelin* mRNA levels trend to increase early after plating, no statistical significance between the time points studied was observed (Fig. 3.7D).

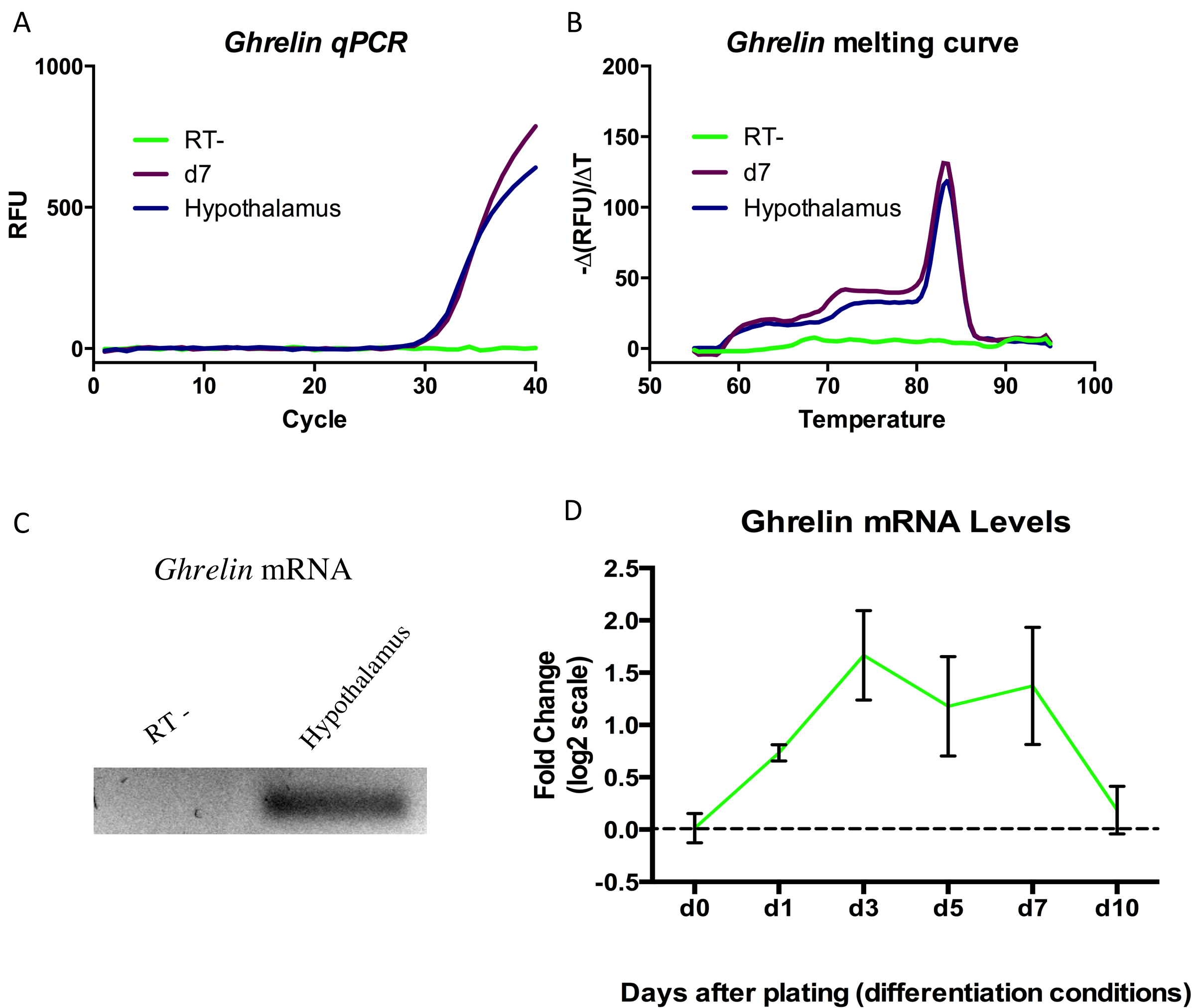


Figure 3.7: Analysis of Ghrelin mRNA expression on DG NPCs derived cells. (A) Relative fluorescence (RFU) and (B) melting curve charts showed that *ghrelin* cDNA is amplified in neurospheres at 7 days (d7) after plating (purple) and in hypothalamic tissue samples (blue). (C) Agarose gel image showing a specific band corresponding to the expected size of the amplification product. (D) Levels of *ghrelin* mRNA trends to increase early after plating.

To analyze whether ghrelin peptide is synthesized in our DG neurosphere cultures, we decided to use an ELISA kit to detect the presence of ghrelin in neurosphere culture media (Fig. 3.8). We observed that the neurosphere cell culture medium contains 10.4 ± 1.5ng/ml of ghrelin. Till our knowledge, this is the first time that synthesis of ghrelin is shown in DG neurosphere cell cultures.

Our results indicate that ghrelin is synthesized by NPCs or derived cells, suggesting that ghrelin system may be relevant for these cells.

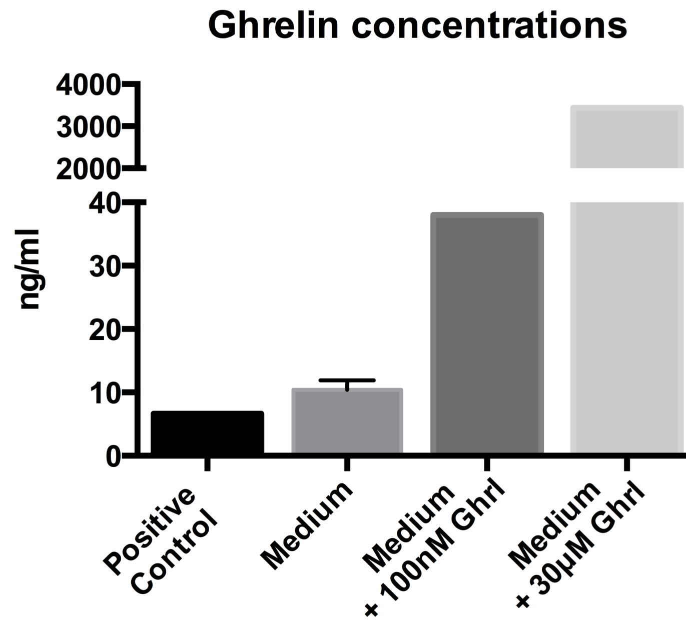


Figure 3.8: Analysis of ghrelin peptide in neurosphere cell cultures. Neurosphere cell culture medium contains 10,4 ± 1,5 ng/ml of ghrelin, detected by ELISA. Three positive controls were used: a cell culture medium provided with the commercial ELISA kit and cell culture media from neurospheres treated with two different dilutions of ghrelin. N=8. Ghrl, ghrelin.

3.5 Effects of exogenous ghrelin on DG neurosphere cultures

A previous study demonstrated that acylated ghrelin has an effect on NPC cultures lacking Ghsr1 expression (Johansson *et al.* 2008). Based on this, we decided to treat our DG neurosphere cultures with acylated ghrelin and check whether, despite of the absence of GHSR1a, our cells still respond to this peptide. We followed two different protocols that have been previously described in the literature to increase proliferation of NPCs (Johansson et al. 2008; Chung et al. 2013). Our results show no significant effect of ghrelin treatments with 100nM (Fig. 3.9A) or 30µM (Fig. 3.9B) in the proportion of proliferative Ki67+ cells, DCX neurons or Nestin+ cells. However, a significant increase in pyknotic bodies, probably reflecting cell death, was observed on neurospheres treated with 30µM of ghrelin.

Our data indicate that the addition of exogenous acylated ghrelin has no effect on cell proliferation or neuronal differentiation on our DG neurosphere cultures.

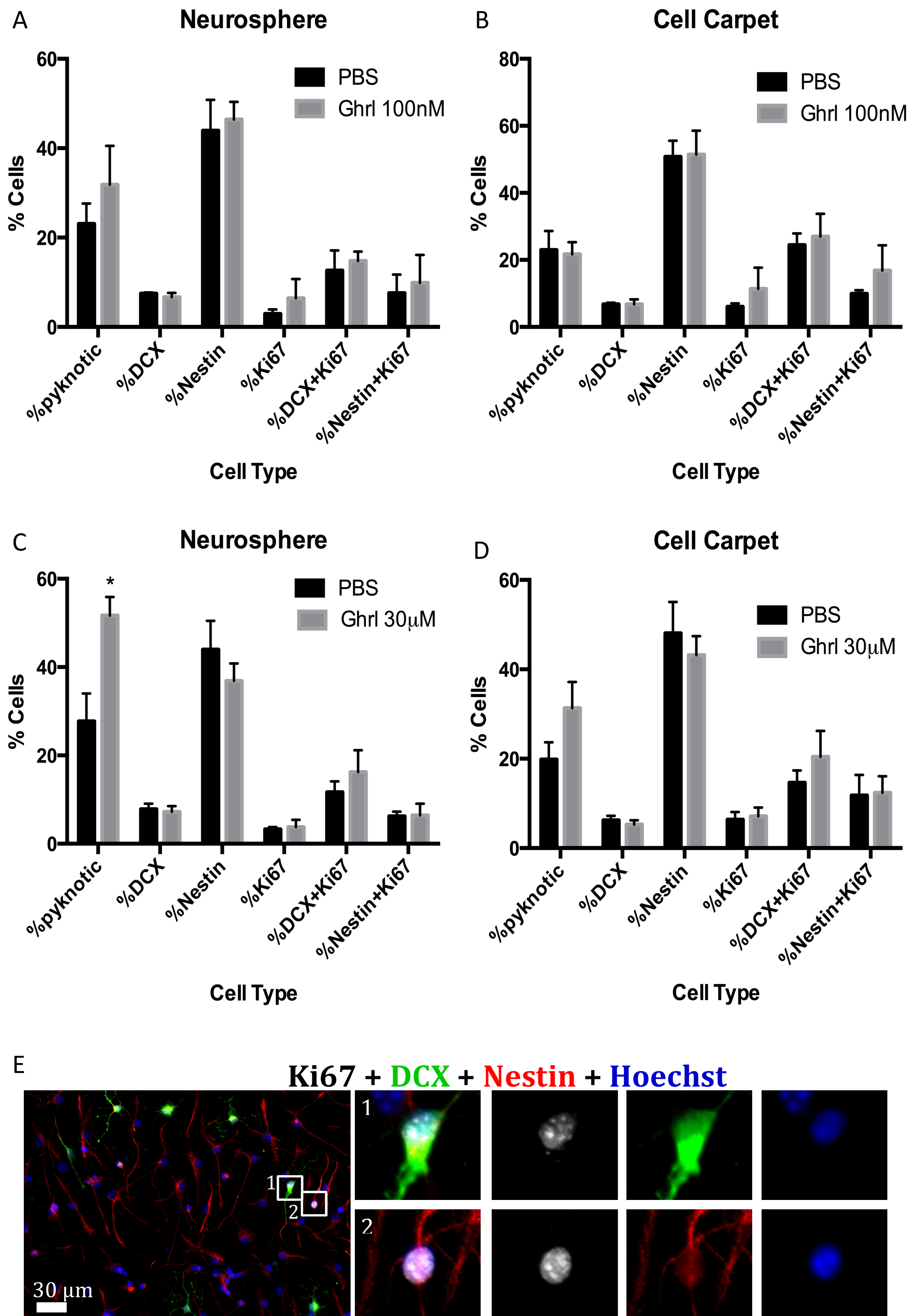

A
Neurosphere
% Cells
PBS
Ghrl 100nM
%pyknotic
%DCX
%Nestin
%Ki67
%DCX+Ki67
%Nestin+Ki67
Cell Type
B
Cell Carpet
% Cells
PBS
Ghrl 100nM
Cell Type
C
Neurosphere
*
PBS
Ghrl 30μM
Cell Type
D
Cell Carpet
PBS
Ghrl 30μM
Cell Type
E
Ki67 + DCX + Nestin + Hoechst
30 μm
1
2

Figure 3.9: Effects of exogenous ghrelin on DG neurospheres culture. Neurospheres were treated with 100nM (A, B) and 30µM (C, D) of ghrelin. No significant effect of ghrelin treatments was observed on DG NPCs cultures in the proportion of Ki67+ cells, DCX neurons or Nestin+ cells. However, a significant increase in pyknotic bodies was observed in neurosphere center after treatment with 30µM. (E) Fluorescence images of DG cultures showing double stained DCX(green)/Ki67(white), and Nestin(red)/Ki67 cells. Cell nuclei were stained with Hoechst 33342 (blue). DCX, doublecortin; Ghrl, ghrelin.

Chapter 4

Discussion

Ghrelin, the orexigenic gut hormone has been widely studied and related with the effect of diet in the control of neural functions. Some studies have described the role of ghrelin in memory and learning, and associated ghrelin with hippocampal adult neurogenesis (Moon *et al.* 2009; Li, E *et al.* 2013a; Li, E *et al.* 2013b; Zhao *et al.* 2014; Moon et al. 2014; Li, E et al. 2014). Ghrelin exerts it effects by binding to its receptor, GHSR-1a (Kojima et al. 1999; Takaya et al. 2000), which is the only functional receptor characterized and identified until now. Importantly, GHSR-1b, the ghrelin truncated receptor, has been described to be mainly present in the ER and to form heterodimers with GHSR-1a, retaining it at the ER membrane and avoiding its translocation to the cell surface where it exerts its function. Till our knowledge, two works has focused on the effect of Ghrelin/GHSR1 system on hippocampal NPCs, demonstrating that ghrelin exert a direct effect in adult rat hippocampal NPCs, leading to an increase in their proliferation. However, none of these works have analyzed the effect of ghrelin on NPCs differentiation.

In the present work, we show that GHSR-1a is absent in neurosphere cell cultures derived from the dentate gyrus. Nevertheless, we observed that another isoform of GHSR1 (probably its truncated isoform GHSR1b) is expressed by neuroprecursor cells (NPCs) and differentiating neurons in these cultures. Furthermore, we provide, for the first time, evidences of the production of ghrelin in DG neurosphere cultures. Finally we observed that the addition of exogenous acylated ghrelin to our cultures of DG neurosphere has no effect on cell proliferation or neuronal differentiation of DG NPCs.

Role of GHSR1 on DG neurosphere cell cultures

Although a previous work has described the presence of GHSR-1a in NPCs (Chung et al. 2013), our cultures present undetectable levels of Ghsr-1a mRNA at different days after neurosphere plating (from day 1 to day 10). Despite of the lack of Ghsr1a mRNA in our DG neurospheres, we detected the expression of Ghsr1 mRNAs containing the exon 2 of murine *Ghsr1* gene. There are some controversies regarding the expression of GHSR-1a on hippocampal NPCs. A Study from Johansson *et al.* showed by qPCR that there is no expression of the ghrelin receptor in adult rat hippocampal progenitor (AHP) cells (Johansson et al. 2008), while Chung *et al.* observed the presence of GHSR-1a in adult rat hippocampal neural stem cells (NSCs) using western blotting and immunocytochemistry (Chung et al. 2013). However, the antibody used by Chung *et al*. was not specific for GHSR-1a (#sc-10362, Santa Cruz Biotechnology), since it recognizes the N-terminal region of GHSR1, being able to detect GHSR-1a and GHSR-1b. Furthermore, we observed that using the same antibody as Chung et al., our western blotting of hypothalamus and hippocampus samples presented several unspecific bands. However, using an antibody (Abcam[1] #ab95250) that recognizes the middle region (150-250 amino acids) of GHSR1, western blotting membranes presented a more clear and restricted band pattern, showing only two bands at 42kDa and at 32kDa (the bands expected for GHSR-1a and GHSR-1b, respectively). Considering this, we cannot exclude the possibility that isoform 1b was the one detected by Chung et al. Meanwhile, in neurosphere samples 5 days after platting, this antibody (Abcam[1] #ab95259) presented a new band with an unexpected size between 42k and 32Da, suggesting the possibility of the existence of a non described GHSR1 variant in our DG neurosphere culture. In agreement, it has been previously proposed the existence of a non described GHSR1 isoform on hippocampal NPCs. This assumption was based on the existence of a binding site for

hexarelin, an analog of ghrelin, in rat AHP cells that lack GHSR-1a expression (Johansson et al. 2008). Several facts suggested that the GHSR1 isoform detected in our neurosphere cultures is indeed GHSR-1b. First, the lower band observed in our western blot membranes corresponds to the size expected for GHSR-1b. Second, when we used the same anti-GHSR1 antibody used for western blot, our immunofluorescent signal mainly appears in the perinuclear region. Importantly, this is a characteristic pattern of proteins located in the endoplasmic reticulum (ER) membrane, and GHSR-1b has been described to be mainly present at the ER (Leung et al. 2007; Chow et al. 2012). Third, the absence of *Ghsr1a* mRNA and existence of mRNA containing GHSR1 exon 2 suggest the presence of a truncated splice variant, like GHSR-1b, in DG neurosphere cultures. The small proportion of cells that express GHSR1 in our cultures (15.87 ± 2%) may indicate that this expression is irrelevant. However, half of the neuronal cells in our cultures exhibited the receptor. These data suggest that the expression of GHSR1 should be important, somehow, for the neuronal population of cells.

Relevance of ghrelin for DG neurosphere cell cultures

Till our knowledge, we are the first to show that ghrelin is produced in cell cultures of DG derived neurosheres. Some studies have suggested that ghrelin is synthesized locally in the hypothalamus (Lu et al. 2002; Cowley et al. 2003) and, curiously, this region also contains a particular neurogenic niche. Whether ghrelin is also produced *in vivo* by cells of the DG neurogenic niche needs to be further explored. Despite what it has been observed in other studies, the addition of exogenous acylated ghrelin to our neurosphere cultures did not induced any significant difference in the proportion of proliferation (Ki67+ cells) or neuronal differentiation. A possible explanation for the lack of effect of ghrelin is the absence of its receptor, GHSR-1a. However, even with no detectable levels of GHSR1a in their hippocampal NPCs cell cultures, Johansson et al. observed that acylated ghrelin increased cell proliferation (Johansson et al. 2008). Another possible explanation for the lack of effect of exogenous ghrelin in our cultures is that *our in vitro* system may be overloaded with endogenous produced ghrelin. In this case, the addition of high levels of ghrelin to the media could be even toxic, as suggested by the increase in the proportion of pyknotic cells when treating cells with 30μM of ghrelin.

Our study reveals that ghrelin system is present in NPC cultures derived from DG. Furthermore, our data points to the possibility of the existence of an endogenous source of ghrelin in the DG neurogenic niche. Our work leaves several important question opened and reveals the necessity of deeping on the analysis of ghrelin system and its interaction with neurogenic cells. Further studies are needed to understand the secondary effects, desired or not desired, of popular habits or interventions that could affect ghrelin system (i.e: maternal malnutrition, gastric bypass, dietary restriction).

Chapter 5

Conclusions

Considering our work hypothesis, the main aims of this study and the results obtained, we conclude that:

1. Dentate gyrus neurosphere cultures do not express GHSR-1a but that, at least, another isoform of this receptor is present in these cultures.

2. GHSR1 is expressed by NPCs and neurons at different stages of maturation in our DG derived neurosphere cultures.

3. Ghrelin is present in the conditioned media of DG differentiating neurosphere cultures.

4. Exogenous acylated-ghrelin treatments does not affect cell proliferation or neuronal differentiation on DG NPCs cultures.

Summarizing, we conclude that ghrelin/GHSR1 system is present in our DG derived neurosphere cell cultures, suggesting that this system may play a direct role in the biology of DG NPCs and derived cells.

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Chapter 4

References

Albarrán-Zeckler, Rosie G., and Roy G. Smith. 2013. "The Ghrelin Receptors (GHS-R1a and GHS-R1b)." *Endocrine Development* 25: 5–15. doi:10.1159/000346042.

Altman, J. 1962. "Are New Neurons Formed in the Brains of Adult Mammals?" *Science (New York, N.Y.)* 135: 1127–28. doi:10.1126/science.135.3509.1127.

Alvarez-Buylla, A, and J M Garcia-Verdugo. 2002. "Neurogenesis in Adult Subventricular Zone." *J Neurosci* 22: 629–34. doi:22/3/629 [pii].

Banks, W A, M Tschop, S M Robinson, and M L Heiman. 2002. "Extent and Direction of Ghrelin Transport across the Blood-Brain Barrier Is Determined by Its Unique Primary Structure." *Journal of Pharmacology and Experimental Therapeutics* 302 (2): 822–27. doi:Unsp 34827/996960 Doi 10.1124/Jpet.102.034827.

Beck, Bernard, Nadine Musse, and Alain Stricker-Krongrad. 2002. "Ghrelin, Macronutrient Intake and Dietary Preferences in Long-Evans Rats." *Biochemical and Biophysical Research Communications* 292 (4): 1031–35. doi:10.1006/bbrc.2002.6737.

Bondolfi, Luca, Florian Ermini, Jefferey M. Long, Donald K. Ingram, and Mathias Jucker. 2004. "Impact of Age and Caloric Restriction on Neurogenesis in the Dentate Gyrus of C57BL/6 Mice." *Neurobiology of Aging* 25: 333–40. doi:10.1016/S0197-4580(03)00083-6.

Brown, Jason, Christiana M. Cooper-Kuhn, Gerd Kempermann, Henriette Van Praag, J??rgen Winkler, Fred H. Gage, and H. Georg Kuhn. 2003. "Enriched Environment and Physical Activity Stimulate Hippocampal but Not Olfactory Bulb Neurogenesis." *European Journal of Neuroscience* 17 (10): 2042–46.

Chartrel, N, R Alvear-Perez, J Leprince, X Iturrioz, A Reaux-Le Goazigo, V Audinot, P Chomarat, et al. 2007. "Comment on 'Obestatin, a Peptide Encoded by the Ghrelin Gene, Opposes Ghrelin's Effects on Food Intake'." *Science (New York, N.Y.)* 315: 766; author reply 766. doi:10.1126/science.1137136.

Chen, Ren. 2012. "Ghrelin Improves Cognitive Function in Mice by Increasing the Production of Insulin-like Growth Factor-I in the Hippocampus." *Nagoya Med. J.* 52: 117–34. http://www.med.nagoya-cu.ac.jp/igak.dir/P117-134chin.pdf.

Chow, Kevin B S, Jingxin Sun, Kit Man Chu, Wing Tai Cheung, Christopher H K Cheng, and Helen Wise. 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: 247–54. doi:10.1016/j.mce.2011.08.034.

Chung, H, E Li, Y Kim, S Kim, and S Park. 2013. "Multiple Signaling Pathways Mediate Ghrelin-Induced Proliferation of Hippocampal Neural Stem Cells." *J Endocrinol* 218 (1): 49–59. doi:10.1530/JOE-13-0045.

Cowley, Michael A., Roy G. Smith, Sabrina Diano, Matthias Tsch??p, Nina Pronchuk, Kevin L. Grove, Christian J. Strasburger, et al. 2003. "The Distribution and Mechanism of Action of Ghrelin in the

CNS Demonstrates a Novel Hypothalamic Circuit Regulating Energy Homeostasis." *Neuron* 37: 649–61. doi:10.1016/S0896-6273(03)00063-1.

Cummings, David E, David S Weigle, R Scott Frayo, Patricia A Breen, Marina K Ma, E Patchen Dellinger, and Jonathan Q Purnell. 2002. "Plasma Ghrelin Levels after Diet-Induced Weight Loss or Gastric Bypass Surgery." *The New England Journal of Medicine* 346 (21): 1623–30. doi:10.1056/NEJMoa012908.

Cummings, David E., Jonathan Q. Purnell, R. Scott Frayo, Karin Schmidova, Brent E. Wisse, and David S. Weigle. 2001. "A Preprandial Rise in Plasma Ghrelin Levels Suggests a Role in Meal Initiation in Humans." *Diabetes* 50 (8): 1714–19. doi:10.2337/diabetes.50.8.1714.

Date, Y, M Kojima, H Hosoda, A Sawaguchi, M S Mondal, T Suganuma, S Matsukura, K Kangawa, and M Nakazato. 2000. "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 (11): 4255–61. doi:Doi 10.1210/En.141.11.4255.

Date, Yukari, Noboru Murakami, Koji Toshinai, Shigeru Matsukura, Akira Niijima, Hisayuki Matsuo, Kenji Kangawa, and Masamitsu Nakazato. 2002. "The Role of the Gastric Afferent Vagal Nerve in Ghrelin-Induced Feeding and Growth Hormone Secretion in Rats." *Gastroenterology* 123: 1120–28. doi:10.1053/gast.2002.35954.

Diano, S, S A Farr, S C Benoit, E C McNay, I da Silva, B Horvath, F S Gaskin, et al. 2006. "Ghrelin Controls Hippocampal Spine Synapse Density and Memory Performance." *Nat Neurosci* 9 (3): 381–88. doi:10.1038/nn1656.

Eriksson, P S, E Perfilieva, T Bjork-Eriksson, A M Alborn, C Nordborg, D A Peterson, and F H Gage. 1998. "Neurogenesis in the Adult Human Hippocampus." *Nat Med* 4 (11): 1313–17. doi:10.1038/3305.

Fontán-Lozano, Angela, José Luis Sáez-Cassanelli, Mari Carmen Inda, Mercedes de los Santos-Arteaga, Sergio Antonio Sierra-Domínguez, Guillermo López-Lluch, José María Delgado-García, and Angel Manuel Carrión. 2007. "Caloric Restriction Increases Learning Consolidation and Facilitates Synaptic Plasticity through Mechanisms Dependent on NR2B Subunits of the NMDA Receptor." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 27 (38): 10185–95. doi:10.1523/JNEUROSCI.2757-07.2007.

Francis, Heather, and Richard Stevenson. 2013. "The Longer-Term Impacts of Western Diet on Human Cognition and the Brain." *Appetite*. doi:10.1016/j.appet.2012.12.018.

Gage, F. H. 2000. "Mammalian Neural Stem Cells." *Science*. doi:10.1126/science.287.5457.1433.

Garthe, A, J Behr, and G Kempermann. 2009. "Adult-Generated Hippocampal Neurons Allow the Flexible Use of Spatially Precise Learning Strategies." *PLoS One* 4 (5): e5464. doi:10.1371/journal.pone.0005464.

Gil-Campos, M, C M Aguilera, R Canete, and A Gil. 2006. "Ghrelin: A Hormone Regulating Food Intake and Energy Homeostasis." *Br J Nutr* 96 (2): 201–26. http://www.ncbi.nlm.nih.gov/pubmed/16923214.

Gillette-Guyonnet, Sophie, and Bruno Vellas. 2008. "Caloric Restriction and Brain Function." *Current*

Opinion in Clinical Nutrition and Metabolic Care 11: 686–92. doi:10.1097/MCO.0b013e328313968f.

Gnanapavan, Sharmilee, Blerina Kola, Stephen A Bustin, Damian G Morris, Patrick McGee, Peter Fairclough, Satya Bhattacharya, Robert Carpenter, Ashley B Grossman, and Márta Korbonits. 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. doi:10.1210/jc.87.6.2988.

Granata, Riccarda, Alessandra Baragli, Fabio Settanni, Francesca Scarlatti, and Ezio Ghigo. 2010. "Unraveling the Role of the Ghrelin Gene Peptides in the Endocrine Pancreas." *Journal of Molecular Endocrinology*. doi:10.1677/JME-10-0019.

Guan, X M, H Yu, O C Palyha, K K McKee, S D Feighner, D J S Sirinathsinghji, R G Smith, L H T VanderPloeg, and A D Howard. 1997. "Distribution of mRNA Encoding the Growth Hormone Secretagogue Receptor in Brain and Peripheral Tissues." *Molecular Brain Research* 48 (1): 23–29. doi:Doi 10.1016/S0169-328x(97)00071-5.

Hers, Ingeborg, Emma E. Vincent, and Jeremy M. Tavaré. 2011. "Akt Signalling in Health and Disease." *Cellular Signalling*. doi:10.1016/j.cellsig.2011.05.004.

Holst, Birgitte, Kristoffer L Egerod, Enrico Schild, Steve P Vickers, Sharon Cheetham, Lars-Ole Gerlach, Laura Storjohann, et al. 2007. "GPR39 Signaling Is Stimulated by Zinc Ions but Not by Obestatin." *Endocrinology* 148: 13–20. doi:10.1210/en.2006-0933.

Hosoda, H, M Kojima, H Matsuo, and K Kangawa. 2000. "Ghrelin and Des-Acyl Ghrelin: Two Major Forms of Rat Ghrelin Peptide in Gastrointestinal Tissue." *Biochemical and Biophysical Research Communications* 279: 909–13. doi:10.1006/bbrc.2000.4039.

Howard, A D, S D Feighner, D F Cully, J P Arena, P A Liberator, C I Rosenblum, M Hamelin, et al. 1996. "A Receptor in Pituitary and Hypothalamus That Functions in Growth Hormone Release." *Science* 273 (5277): 974–77. doi:Doi 10.1126/Science.273.5277.974.

Hubina, Erika, Miklós Góth, and Márta Korbonits. 2005. "Ghrelin--a Hormone with Multiple Functions." *Orvosi Hetilap* 146: 1345–51. doi:10.1016/j.yfrne.2004.03.002.

Jeffery, P L, A C Herington, and L K Chopin. 2003. "The Potential Autocrine/paracrine Roles of Ghrelin and Its Receptor in Hormone-Dependent Cancer." *Cytokine & Growth Factor Reviews* 14 (2): 113–22. doi:Doi 10.1016/S1359-6101(02)00089-8.

Johansson, Inger, Silvia Destefanis, N David Aberg, Maria A I Aberg, Klas Blomgren, Changlian Zhu, Corrado Ghè, et al. 2008. "Proliferative and Protective Effects of Growth Hormone Secretagogues on Adult Rat Hippocampal Progenitor Cells." *Endocrinology* 149: 2191–99. doi:10.1210/en.2007-0733.

Kit-Man Chu, Kevin B.S. Chow, Po-Ki Leung, Pui-Ngan Lau, Chi-Bun Chan, Christopher H.K. Cheng, and Helen Wise. 2007. "Over-Expression of the Truncated Ghrelin Receptor Polypeptide Attenuates the Constitutive Activation of Phosphatidylinositol-Specific Phospholipase C by Ghrelin Receptors but Has No Effect on Ghrelin-Stimulated Extracellular Signal-Regulated Kinase 1/2 Ac." *International Journal of Biochemestry and Cell Biology* 39: 752–64. http://www.ncbi.nlm.nih.gov/pubmed/?term=Over-

expression+of+the+truncated+ghrelin+receptor+polypeptide+attenuates+the+constitutive+activation+of+phosphatidylinositol-specific+phospholipase+C+by+ghrelin+receptors+but+has+no+effect+on+ghrelin-stimulated+extracellular+signal-regulated+kinase+1%2F2+activity.

Klok, M D, S Jakobsdottir, and M L Drent. 2007. "The Role of Leptin and Ghrelin in the Regulation of Food Intake and Body Weight in Humans: A Review." *Obesity Reviews* 8 (1): 21–34. doi:Doi 10.1111/J.1467-789x.2006.00270.X.

Kojima, M, H Hosoda, Y Date, M Nakazato, H Matsuo, and K Kangawa. 1999. "Ghrelin Is a Growth-Hormone-Releasing Acylated Peptide from Stomach." *Nature* 402 (6762): 656–60. doi:Doi 10.1038/45230.

Kojima, M, and K Kangawa. 2005. "Ghrelin: Structure and Function." *Physiological Reviews* 85 (2): 495–522. doi:Doi 10.1152/Physrev.00012.2004.

Korbonits, M, A P Goldstone, M Gueorguiev, and A B Grossman. 2004. "Ghrelin - a Hormone with Multiple Functions." *Front Neuroendocrinol* 25 (1): 27–68. doi:Doi 10.1016/J.Yfrne.2004.03.002.

Lauwers, Erwin, Bart Landuyt, Lutgarde Arckens, Liliane Schoofs, and Walter Luyten. 2006. "Obestatin Does Not Activate Orphan G Protein-Coupled Receptor GPR39." *Biochemical and Biophysical Research Communications* 351: 21–25. doi:10.1016/j.bbrc.2006.09.141.

Lee, Jaewon, Kim B. Seroogy, and Mark P. Mattson. 2002. "Dietary Restriction Enhances Neurotrophin Expression and Neurogenesis in the Hippocampus of Adult Mice." *Journal of Neurochemistry* 80: 539–47. doi:10.1046/j.0022-3042.2001.00747.x.

Leidy, Heather J, Kelly A Dougherty, Brian R Frye, Kristin M Duke, and Nancy I Williams. 2007. "Twenty-Four-Hour Ghrelin Is Elevated after Calorie Restriction and Exercise Training in Non-Obese Women." *Obesity (Silver Spring, Md.)* 15 (2): 446–55. doi:10.1038/oby.2007.542.

Leonard, W J, and J J O'Shea. 1998. "Jaks and STATs: Biological Implications." *Annual Review of Immunology* 16: 293–322. doi:10.1146/annurev.immunol.16.1.293.

Leung, Po K., K. B S Chow, Pui Ngan Lau, Kit M. Chu, Chi B. Chan, C. H K Cheng, and Helen Wise. 2007. "The Truncated Ghrelin Receptor Polypeptide (GHS-R1b) Acts as a Dominant-Negative Mutant of the Ghrelin Receptor." *Cellular Signalling* 19: 1011–22. doi:10.1016/j.cellsig.2006.11.011.

Levenson, Cathy W, and Nicholas J Rich. 2007. "Eat Less, Live Longer? New Insights into the Role of Caloric Restriction in the Brain." *Nutrition Reviews* 65: 412–15. doi:10.1111/j.1753-4887.2007.tb00319.x.

Li, E, Y Kim, S Kim, T Sato, M Kojima, and S Park. 2014. "Ghrelin Stimulates Proliferation, Migration and Differentiation of Neural Progenitors from the Subventricular Zone in the Adult Mice." *Exp Neurol* 252: 75–84. doi:10.1016/j.expneurol.2013.11.021.

Li, Endan, Hyunju Chung, Y Kim, Dh Kim, and Jh Ryu. 2012. "Ghrelin Directly Stimulates Adult Hippocampal Neurogenesis: Implications for Learning and Memory." *Endocrine …* 60 (6): 781–89. doi:10.1507/endocrj.

Li, Endan, Yumi Kim, Sehee Kim, and Seungjoon Park. 2013. "Ghrelin-Induced Hippocampal

Neurogenesis and Enhancement of Cognitive Function Are Mediated Independently of GH/IGF-1 Axis: Lessons from the Spontaneous Dwarf Rats." *Endocrine Journal*. doi:10.1507/endocrj.

Liu, Boyang, Edwin A. Garcia, and Márta Korbonits. 2011. "Genetic Studies on the Ghrelin, Growth Hormone Secretagogue Receptor (GHSR) and Ghrelin O-Acyl Transferase (GOAT) Genes." *Peptides*. doi:10.1016/j.peptides.2011.09.006.

Lomenick, Jefferson P., Maria S. Melguizo, Sabrina L. Mitchell, Marshall L. Summar, and James W. Anderson. 2009. "Effects of Meals High in Carbohydrate, Protein, and Fat on Ghrelin and Peptide YY Secretion in Prepubertal Children." *Journal of Clinical Endocrinology and Metabolism* 94 (11): 4463–71. doi:10.1210/jc.2009-0949.

Lu, S., J. L. Guan, Q. P. Wang, K. Uehara, S. Yamada, N. Goto, Y. Date, et al. 2002. "Immunocytochemical Observation of Ghrelin-Containing Neurons in the Rat Arcuate Nucleus." *Neuroscience Letters* 321 (3): 157–60. doi:10.1016/S0304-3940(01)02544-7.

Maalouf, Marwan, Jong M. Rho, and Mark P. Mattson. 2009. "The Neuroprotective Properties of Calorie Restriction, the Ketogenic Diet, and Ketone Bodies." *Brain Research Reviews*. doi:10.1016/j.brainresrev.2008.09.002.

Mirescu, Christian, and Elizabeth Gould. 2006. "Stress and Adult Neurogenesis." *Hippocampus*.

Moon, M, M Y Cha, and I Mook-Jung. 2014. "Impaired Hippocampal Neurogenesis and Its Enhancement with Ghrelin in 5XFAD Mice." *J Alzheimers Dis* 41 (1): 233–41. doi:10.3233/jad-132417.

Moon, M, S Kim, L Hwang, and S Park. 2009. "Ghrelin Regulates Hippocampal Neurogenesis in Adult Mice." *Endocr J* 56: 525–31. doi:JST.JSTAGE/endocrj/K09E-089 [pii].

Morrison, J H, and P R Hof. 1997. "Life and Death of Neurons in the Aging Brain." *Science (New York, N.Y.)* 278 (5337): 412–19. doi:10.1126/science.278.5337.412.

Olson, Andrea K., Brennan D. Eadie, Carl Ernst, and Brian R. Christie. 2006. "Environmental Enrichment and Voluntary Exercise Massively Increase Neurogenesis in the Adult Hippocampus via Dissociable Pathways." *Hippocampus*. doi:10.1002/hipo.20157.

Park, June Hee, Zachary Glass, Kasim Sayed, Tatyana V. Michurina, Alexander Lazutkin, Olga Mineyeva, Dmitry Velmeshev, Walter F. Ward, Arlan Richardson, and Grigori Enikolopov. 2013. "Calorie Restriction Alleviates the Age-Related Decrease in Neural Progenitor Cell Division in the Aging Brain." *European Journal of Neuroscience* 37: 1987–93. doi:10.1111/ejn.12249.

Petersenn, S, A C Rasch, M Penshorn, F U Beil, and H M Schulte. 2001. "Genomic Structure and Transcriptional Regulation of the Human Growth Hormone Secretagogue Receptor." *Endocrinology* 142 (6): 2649–59. doi:Doi 10.1210/En.142.6.2649.

Piatti, Verónica C, Laura a Ewell, and Jill K Leutgeb. 2013. "Neurogenesis in the Dentate Gyrus: Carrying the Message or Dictating the Tone." *Frontiers in Neuroscience* 7: 50. doi:10.3389/fnins.2013.00050.

Ribeiro, Luís F, Tatiana Catarino, Sandra D Santos, Marion Benoist, J Fiona van Leeuwen, José a Esteban, and Ana Luísa Carvalho. 2014. "Ghrelin Triggers the Synaptic Incorporation of AMPA

Receptors in the Hippocampus." *Proceedings of the National Academy of Sciences of the United States of America* 111 (1): E149–58. doi:10.1073/pnas.1313798111.

Romero, Amparo, Henriette Kirchner, Kristy Heppner, Paul T Pfluger, Matthias H Tschöp, and Ruben Nogueiras. 2010. "GOAT: The Master Switch for the Ghrelin System?" *European Journal of Endocrinology / European Federation of Endocrine Societies* 163: 1–8. doi:10.1530/EJE-10-0099.

Roskoski, Robert. 2012. "ERK1/2 MAP Kinases: Structure, Function, and Regulation." *Pharmacological Research : The Official Journal of the Italian Pharmacological Society* 66 (2): 105–43. doi:10.1016/j.phrs.2012.04.005.

Sakata, Ichiro, and Takafumi Sakai. 2010. "Ghrelin Cells in the Gastrointestinal Tract." *International Journal of Peptides*. doi:10.1155/2010/945056.

Sato, Takahiro, Yuki Nakamura, Yuki Shiimura, Hideko Ohgusu, Kenji Kangawa, and Masayasu Kojima. 2012. "Structure, Regulation and Function of Ghrelin." *Journal of Biochemistry*. doi:10.1093/jb/mvr134.

Schoenfeld, Timothy J., and Elizabeth Gould. 2012. "Stress, Stress Hormones, and Adult Neurogenesis." *Experimental Neurology*. doi:10.1016/j.expneurol.2011.01.008.

Seib, D. R. and Martin-Villalba, A. (2013). *In vivo* Neurogenesis. *Bio-protocol* 3(15): e841. http://www.bio-protocol.org/e841

Stangl, Doris, and Sandrine Thuret. 2009. "Impact of Diet on Adult Hippocampal Neurogenesis." *Genes and Nutrition*. doi:10.1007/s12263-009-0134-5.

Stranahan, Alexis M., Eric D. Norman, Kim Lee, Roy G. Cutler, Richard S. Telljohann, Josephine M. Egan, and Mark P. Mattson. 2008. "Diet-Induced Insulin Resistance Impairs Hippocampal Synaptic Plasticity and Cognition in Middle-Aged Rats." *Hippocampus* 18 (11): 1085–88. doi:10.1002/hipo.20470.

Takaya, K, H Ariyasu, N Kanamoto, H Iwakura, A Yoshimoto, M Harada, K Mori, et al. 2000. "Ghrelin Strongly Stimulates Growth Hormone (GH) Release in Humans." *Journal of Clinical Endocrinology & Metabolism* 85 (12): 4908–11. doi:Doi 10.1210/Jc.85.12.4908.

Tang, Sheng Qiu, Qing Yan Jiang, Yong Liang Zhang, Xiao Tong Zhu, Gang Shu, Ping Gao, Ding Yuan Feng, Xiu Qi Wang, and Xiao Ying Dong. 2008. "Obestatin: Its Physicochemical Characteristics and Physiological Functions." *Peptides*. doi:10.1016/j.peptides.2008.01.012.

Tanti, Arnaud, Quentin Rainer, Frederic Minier, Alexandre Surget, and Catherine Belzung. 2012. "Differential Environmental Regulation of Neurogenesis along the Septo-Temporal Axis of the Hippocampus." *Neuropharmacology* 63 (3): 374–84.

Torres-Aleman, Ignacio. 2010. "Toward a Comprehensive Neurobiology of IGF-I." *Developmental Neurobiology*. doi:10.1002/dneu.20778.

Weigle, David S., David E. Cummings, Patricia D. Newby, Patricia A. Breen, R. Scott Frayo, Colleen C. Matthys, Holly S. Callahan, and Jonathan Q. Purnell. 2003. "Roles of Leptin and Ghrelin in the Loss of Body Weight Caused by a Low Fat, High Carbohydrate Diet." *Journal of Clinical*

Endocrinology and Metabolism 88 (4): 1577–86. doi:10.1210/jc.2002-021262.

Williams, Diana L, Harvey J Grill, David E Cummings, and Joel M Kaplan. 2003. “Vagotomy Dissociates Short- and Long-Term Controls of Circulating Ghrelin.” *Endocrinology* 144: 5184–87. doi:10.1210/en.2003-1059.

Witte, A V, M Fobker, R Gellner, S Knecht, and A Flöel. 2009. *Caloric Restriction Improves Memory in Elderly Humans. Proceedings of the National Academy of Sciences of the United States of America*. Vol. 106. doi:10.1073/pnas.0808587106.

Wu, A., R. Molteni, Z. Ying, and F. Gomez-Pinilla. 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 (2): 365–75. doi:10.1016/S0306-4522(03)00154-4.

Yang, Hyunwon, Yun Hee Youm, Chiaki Nakata, and Vishwa Deep Dixit. 2007. “Chronic Caloric Restriction Induces Forestomach Hypertrophy with Enhanced Ghrelin Levels during Aging.” *Peptides* 28 (10): 1931–36. doi:10.1016/j.peptides.2007.07.030.

Zhang, J V, P G Ren, O Avsian-Kretchmer, C W Luo, R Rauch, C Klein, and A J W Hsueh. 2005. “Obestatin, a Peptide Encoded by the Ghrelin Gene, Opposes Ghrelin’s Effects on Food Intake.” *Science* 310 (5750): 996–99. doi:Doi 10.1126/Science.1117255.

Zhao, Chunmei, Wei Deng, and Fred H. Gage. 2008a. “Mechanisms and Functional Implications of Adult Neurogenesis.” *Cell*. doi:10.1016/j.cell.2008.01.033.

———. 2008b. “Mechanisms and Functional Implications of Adult Neurogenesis.” *Cell*.

Zhao, Z., H. Liu, K. Xiao, M. Yu, L. Cui, Q. Zhu, R. Zhao, G. D. Li, and Y. Zhou. 2014. “Ghrelin Administration Enhances Neurogenesis but Impairs Spatial Learning and Memory in Adult Mice.” *Neuroscience* 257: 175–85. doi:10.1016/j.neuroscience.2013.10.063.

Zigman, J M, J E Jones, C E Lee, C B Saper, and J K Elmquist. 2006. “Expression of Ghrelin Receptor mRNA in the Rat and the Mouse Brain. (vol 494, Pg 528, 2006).” *Journal of Comparative Neurology* 499 (4): 690. doi:Doi 10.1002/Cne.21171.