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A NEUROENDOCRINE STRATEGY TO DELAY AGING

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A NEUROENDOCRINE STRATEGY TO DELAY AGING

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in text and are listed in the included references.

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Abstract

Aging is an ongoing process that cannot be stopped, being characterized by a progressive loss of physiological integrity. Hallmarks of aging can be divided in 3 major categories: primary, antagonistic and integrative. Hypothalamus is known for is key role in the endocrine system, being responsible to regulate growth, development, reproduction, metabolism, systemic aging, and ultimately lifespan control.

Ghrelin, known as the "hunger hormone", plays not only a major role in food intake but also in whole-body metabolism. With this investigation, we aimed to evaluate the effect of subcutaneous injection of ghrelin in aged mice. For that, we performed 2 kinds of treatment: a short-term treatment, where animals were submitted to 1-month administration of ghrelin and a gong-term treatment, where animals were submitted to a 2-month treatment. We hypothesise that continuous administration of ghrelin in mice will improve several aging hallmarks.

We observed that ghrelin treatment induces less stress and anxiety in mice. Plus, treated mice presented higher weight loss, but improved BAT, WAT and pancreatic functions. In BAT, treated mice have less signs of adipocytes death. In pancreas, treated mice have a tendency of having larger pancreatic area and less dead cells. In addition, ghrelin treatment significantly decreased hypothalamus inflammation.

We conclude that ghrelin treatment can improve aging hallmarks such as mitochondrial dysfunction, proteostasis and nutrient sensing. This way, ghrelin can be studied in the future as a potential therapeutic approach to extend lifespan.

Keywords: Aging ; hypothalamus; aging mechanisms; proteostasis ghrelin; lifespan

Resumo

O envelhecimento é um processo contínuo, caracterizado pela perda progressiva da integridade fisiológica, podendo assim aumentar o risco de desenvolver patologias relacionadas com a idade como o cancro, diabetes, doenças cardiovasculares e doenças neurodegenerativas. Os fatores que influenciam o envelhecimento podem ser divididos em 3 categorias principais: i) fatores primários, que têm um impacto diretamente negativo no organismo; ii) fatores antagónicos, em que o seu impacto no organismo depende diretamente da intensidade desse fator; e iii) fatores integrativos, que são os fatores que afetam a homeostasia corporal.

O hipotálamo desempenha uma função de extrema importância no sistema endócrino, tornando-o responsável pela regulação do crescimento, desenvolvimento, reprodução, metabolismo, envelhecimento sistémico e longevidade.

Embora seja apelidada de "hormona da fome", papel da grelina é muito mais amplo do que a indução da ingestão de alimentos. Por exemplo, no que concerne à adiposidade, a grelina, por um lado, induz a acumulação de ácidos gordos e, por outro lado, inibe a sua oxidação. No cérebro, a grelina atua no hipocampo e melhora a capacidade de memória e aprendizagem, uma vez que tem um efeito neuro-protetor. A grelina tem também função regulatória a nível cardiovascular, ósseo, gastrointestinal, metabolismo da glucose, do timo e do ciclo circadiano.

Com este trabalho, o nosso objetivo é avaliar o efeito da administração subcutânea de grelina em murganhos envelhecidos. Para isso, fizemos 2 tipos de tratamentos: tratamento de curto prazo, em que a grelina foi administrada diariamente durante um mês, e um tratamento de longo prazo, em que a grelina foi administrada diariamente durante 2 meses. A nossa hipótese é que a administração de grelina pode atrasar os principais fatores que levam ao envelhecimento.

Após o tratamento, observámos que o grupo experimental tratado com grelina apresentou menos stress e menos ansiedade face ao grupo controlo. Contrariamente à literatura, observámos que ambos os tratamentos com grelina (curto prazo e longo prazo) induzem perda de massa corporal nos murganhos. Estas observações podem, no entanto, estar relacionadas com a sua avançada idade. Verificámos também que esta perda de massa corporal não está relacionada com a quantidade de comida ingerida, uma vez que esta foi semelhante em ambos os grupos. Estes dados sugerem que a grelina pode influenciar a regulação da massa corporal sem interferir no apetite. No tecido adiposo castanho, observamos que a grelina reduz o número de adipócitos mortos, sugerindo assim que a grelina poderá melhorar a função deste tecido. No tecido adiposo branco, observamos que a área dos adipócitos é significativamente superior nos ratinhos sujeitos ao tratamento. No pâncreas, os ratinhos tratados apresentaram não só uma maior área de ilhas pancreáticas, mas também um menor número de células mortas nas mesmas. Sabendo a importância do pâncreas na regulação da glucose, estes dados sugerem que a grelina poderá desempenhar um papel importante na prevenção de doenças relacionadas com a desregulação da homeostasia da glucose, nomeadamente na diabetes. A nível molecular, no hipotálamo, observámos que o tratamento é capaz de aumentar a expressão de mRNA de genes orexigénicos, nomeadamente os recetores NPY 1 e 2 e o AgRP. Observámos também uma diminuição da expressão do gene anorexigénico POMC. Em relação à inflamação, verificámos que os ratinhos sujeitos ao tratamento apresentaram uma diminuição significativa na expressão de TNF-α, sugerindo assim que que a grelina é capaz de reduzir a sua inflamação.

A administração continua de grelina pode, de facto, melhorar os fatores que levam ao envelhecimento corporal. O facto de no tecido adiposo castanho e no pâncreas existir um número inferior de células mortas poderá ser explicado por uma melhoria na proteostasia mitocondrial através da administração de grelina. Para além disso, alguns estudos demonstraram que a inflamação no hipotálamo está relacionada com a perda da proteostasia. Estes dados sugerem que a grelina poderá abrandar o envelhecimento provocado pela

acumulação de dano celular através do restauro da proteostasia. Verificámos também que os murganhos tratados mostraram uma melhoria da função pancreática, sugerindo assim uma melhoria na regulação da insulina e, por sua vez, na regulação da glucose.

Em suma, um tratamento de grelina poderá melhorar o metabolismo e a homeostasia corporal, em especial nos principais fatores que induzem o envelhecimento. No entanto, estudos e análises adicionais são necessários para melhor compreender a sua ação. Desta forma, a grelina poderá uma possível abordagem terapêutica para aumentar a longevidade.

Palavras-chave: Envelhecimento; hipotálamo; mecanismos de envelhecimento; proteostasia; grelina; longevidade

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List of abbreviations

ABCG1 – ATP-Binding Cassette sub-family G member 1

ACC – Acetyl-Coenzyme-A Carboxylase

ACTH – Adrenocorticotropic Hormone

AD – Alzheimer's Disease

AgRP – Agouti-Related Protein

AHN – Adult Hippocampal Neurogenesis

AMP – Adenosine Monophosphate

AMPK – 5'-Adenosine-Monophosphate-Activated Protein Kinase

APS – Ammonium Persulfate

APT1 – Acyl-Protein Thioesterase 1

ARC – Arcuate Nucleus

Arg23 – Arginine 23

Arg51 – Arginine 51

ATP – Adenosine Triphosphate

BAT – Brown Adipose Tissue

BBB – Blood Brain Barrier

BChE – Butyrylcholinesterase

BDNF – Brain Derived Neurotrophic Factor

BSA – Bovine Serum Albumin

cAMP – Cyclic Adenosine Monophosphate

CART – Cocaine- and Amphetamine-Regulated Transcript

CBMR – Centre for Biomedical Research

C-EBPα – CCAAT-Enhancer-Binding Protein α

CNS – Central Nervous System

CREB – Cyclic-Adenosine-Monophosphate Response Element-Binding Protein

Ct – Cycle Threshold

DGAT1 – Diacylglycerol O-Acyltransferase 1

DNA – Deoxyribonucleic Acid

DTT – Dithiothreitol

FELASA – Federation of Laboratory Animal Science Associations

GDP – Guanosine Diphosphate

GH – Growth Hormone

GHRP-6 – Growth Hormone Releasing Peptide 6

GHSR – Growth Hormone Secretagogue Receptor

GnRH – Gonadotropin-Releasing Hormone

GOAT – Ghrelin O-Acyl Transferase

GPCR – G-Protein Coupled Receptor

GPR83 – Probable G-Protein Coupled Receptor 83

GTP – Guanosine Triphosphate

GTT – Glucose Tolerance Test

HCl – Hydrochloric Acid

HD – Huntington's Disease

HDL – High Density Lipoprotein

HtNSC – Hypothalamic Neural Stem Cell

IGF-1 – Insulin-Like Growth Factor 1

IKK-β – IkB Kinase-β

ITT – Insulin Tolerance Test

IV – Intravenous

KNDy – Kisspeptin, Neurokinin B and Dynorphin A

LXRα – Liver X Receptor Alpha

MAPK – Mitogen Activated Protein Kinase

MBOAT – Membrane O-Acyl Transferase

MC3R – Melanocortin Receptor 3

MC4R – Melanocortin Receptor 4

MSH – Melanocyte-Stimulating Hormone

mTOR – mammalian Target of rapamycin

NaCl – Sodium Chloride

NaOH – Sodium Hydroxide

NF-kB – Nuclear Factor kappa-light-chain-enhancer of activated B cells

NPY – Neuropeptide Y

NSC – Neural Stem cell

PC 1/3 – Prohormone Convertase 1/3

PD – Parkinson's Disease

PI3K – Phosphoinositide-3-Kinase

POMC – Pro-Opiomelanocortin

PVDF – Polyvinylidene Fluoride Membranes

qRT-PCR – Quantitative Real Time Polymerase Chain Reaction

ROS – Reactive Oxygen Species

SDS – Sodium Dodecyl Sulfate

SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide

SEM – Standard Error of Mean

Ser3 – Serine 3

SNS – Sympathetic Nervous System

TBS-T – Tris-Buffered Saline-Tween

TEMED – Tetramethyl-Ethylene-Diamine

TNF-α – Tumor Necrosis Factor α

UCP1 – Uncoupling Protein 1

UCP2 – Uncoupling Protein 2

UPS – Ubiquitin-Proteasome System

WAT – White Adipose Tissue

WB – Western-Blot

B3-AR – β3-Adrenergic Receptor

Introduction

Hallmarks of aging

Aging is an ongoing process that we cannot stop and it is characterized by a progressive loss of physiological integrity, leading to an increased risk of major human pathologies like cancer, diabetes, cardiovascular disorders and neurodegenerative diseases (Lopez-Otin, Blasco et al. 2013). The hallmarks of aging can be divided in 3 major categories (fig. 1): i) primary hallmarks, which have a direct negative effect on the body like genomic instability, mitochondrial dysfunction, telomere loss, epigenetic alterations and defective proteostasis; ii) antagonistic hallmarks, where its effect depends on its intensity. At low levels can be beneficial, but in high levels can be harmful, including for example, cellular senescence and nutrient sensing; and iii) integrative hallmarks, which are the hallmarks that directly affects homeostasis, such as altered intercellular communication and stem cell exhaustion.

Figure 1*- The hallmarks of aging divided in three major categories: Primary hallmarks (Altered intercellular communication, telomere attrition, epigenetic alterations, loss of proteostasis and mitochondrial dysfunction), Antagonistic Hallmarks (Cellular senescence and deregulated nutrient sensing) and Integrative Hallmarks (genomic instability and stem cell exhaustion. (Image adapted from (Lopez-Otin, Blasco et al. 2013)).*

The progressive loss of physiological integrity observed in the aging process might be due to genomic instability, where DNA damage accumulation together with a faulty DNA repair mechanism cause premature aging diseases like Progeria. Genomic instability can be due to both intrinsic factors, like reactive oxygen species (ROS) and replication errors, and extrinsic factors like chemical compounds (Hoeijmakers 2009).

Mitochondria is the organelle responsible for the production of adenosine triphosphate (ATP) cell supply, but is also involved in other important cellular mechanisms like cell cycle, cell growth and differentiation and signalling (McBride, Neuspiel et al. 2006). Mitochondrial dysfunction occurs progressively during age, therefore decreasing efficacy of the respiratory chain (reduction of ATP generation) and, in turn, increasing electron leakage and the production of ROS causing more mitochondrial deterioration and global cellular damage (Green, Galluzzi et al. 2011).

Telomeres are a repetitive region of chromosomes, located at both ends, which confers protection from deterioration or fusion with other chromosomes. Telomeres are shortened in each DNA replication because telomerase (the enzyme responsible for extension of telomeres), is not present in most somatic cell (Cong, Wright et al. 2002). This absence of telomerase leads to cellular senescence and death, when the telomeres become too short (Olovnikov 1996). On the other hand, this shortening can cause loss of vital genetic information or mutations, since the chromosome is no longer protected. The fact that reversing telomere shortening in mice prolongs mice life span (Jaskelioff, Muller et al. 2011), clearly proves that telomere shortening is, in fact, an important hallmark of aging

Epigenetic alterations involving alterations in DNA methylation patterns, post-translational modifications of histones and chromatin remodelling can also have a role in age-related syndromes. These alterations are primary due to environmental factors (Talens, Christensen et al. 2012), inducing alterations in gene expression, which underlies and abnormal cellular functioning and, ultimate leads to cell death.

Loss of proteostasis is another hallmark of aging , as it is essential for the cell to keep not only a correct protein fold and trafficking, but also a fully functional mechanism of protein disposal and degradation (Koga, Kaushik et al. 2011). Proteostasis starts in ribosome, during translation. A correct peptide synthesis is essential to allow a correct folding and, consequently, forming a functional protein. Molecular chaperones are also very important in order to maintain proteostasis, being responsible for protein assembly or disassembly (Kim, Hipp et al. 2013), and their role begin as soon as the peptide leave the ribosome inducing protein folding (Vabulas, Raychaudhuri et al. 2010). Once proteins are no longer needed in the body, they need to be discarded and, to accomplish that, is necessary that protein degradation machinery works properly. Protein disposal is also necessary when they are in a misfolded or unfolded state. Recent reports have shown that loss of proteostasis might be correlated with an inflammatory state of the hypothalamus (Ignacio-Souza, Bombassaro et al. 2014). Also, constitutive expression of misfolded proteins, potentially forming aggregates, may lead to age-related diseases like Alzheimer's and Parkinson's disease and cataracts (Powers, Morimoto et al. 2009). Protein disposal is performed by the ubiquitin-proteasome system (UPS) (Chondrogianni and Gonos 2012). First, ligases E1, E2 and E3 covalently attach ubiquitin protein to the target protein, thus tagging the protein to degradation (Finley 2009). This attachment changes the stability and localization of the target protein and, therefore, changing the stability of the target protein (Pickart and Eddins 2004). Then, a multicatalytic proteolytic particle, called proteasome, recognize tagged proteins and degrade them (Glickman and Ciechanover 2002). Inhibition of this complex (or some of its components) cause homeostatic instability, thus leading to cellular dysfunction and ultimately cell death (Chondrogianni and Gonos 2012). UPS impairment is related to neurodegenerative disorders like Alzheimer's and Parkinson's diseases (Paul 2008) and occurs naturally during aging process (Chondrogianni and Gonos 2005). Autophagy is another mechanism to degrade proteins, both soluble and aggregates and contributes to basal cellular homeostasis (Choi, Ryter et al. 2013). Autophagy is a survival mechanism that happens

under stress conditions, with the goal of maintaining cellular integrity (Ravikumar, Sarkar et al. 2010). This goal is achieved by regenerating cellular metabolites and clearing cellular debris (Mizushima, Levine et al. 2008), constituting an antiaging process (Rubinsztein, Marino et al. 2011). It is well established that autophagy plays a very important role in neurodegenerative diseases, since autophagy is dysregulated in these disorders (Wong and Cuervo 2010). For example, patients suffering from Alzheimer's Disease (AD) have an accelerated autophagosomes accumulation (Ma, Huang et al. 2010). Plus, faulty autophagy proteins enhance neurodegeneration due to protein aggregates (Hara, Nakamura et al. 2006). Thus, autophagy plays a major role in preventing cell death (Choi, Ryter et al. 2013). It has been reported that both excessive autophagy and impaired autophagy are associated with apoptosis (Ravikumar, Sarkar et al. 2010). Autophagy also plays a crucial role during bacterial and viral infections, adaptive immune response, infectious diseases, ((Levine, Mizushima et al. 2011), (Deretic and Levine 2009)), cancer progression and initiation (Ravikumar, Sarkar et al. 2010), cardiovascular diseases (Kirshenbaum 2012), metabolic diseases (Singh, Kaushik et al. 2009), and pulmonary diseases (Monick, Powers et al. 2010). Cellular senescence, defined as stable arrest of the cell cycle, prevents the propagation of damaged cells and a failure in inducing cellular senescence will allow spreading of damage cells. Cellular senescence is beneficial to the well-functioning of the body, but an exacerbate rate of senescence will promote premature aging (Lopez-Otin, Blasco et al. 2013). The replacement of senescent cells with stem cells is essential for the tissue to continue playing its role, and a failure in inducing this process leads to a senescent cells accumulation, instigating tissue aging. It was shown that lifespan can be extended if these senescent cells are removed (Baker, Wijshake et al. 2011).

Nutrient sensing deregulation is probably the most conserved aging control hallmark, where the insulin and insulin-like growth factor 1 (IGF-1) pathway plays a major role. Sighting to minimise cell growth, thus preventing systemic damage, a decrease on the insulin and IGF-1 pathway often occurs during normal aging (Schumacher, van der Pluijm et al. 2008). Controversy, a constitutive deregulated nutrient sensing mechanism leads to extended lifespan in mice (Fontana, Partridge et al. 2010). This occurs because the metabolism and cell growth are slower, decreasing systemic cell damage.

Altered intercellular communication also occurs during aging process due to the increasing of inflammatory reactions and failure of immune system response, thus, neurohormonal signalling tends to be deregulated (Lopez-Otin, Blasco et al. 2013). A study found that decreasing inflammatory state can increase lifespan (Strong, Miller et al. 2008).

Stem cell exhaustion, where the regenerative potential of tissue is decreased or ended, is one of the most important aging hallmarks. DNA damage accumulation, telomere shortening, and other age-related damage can cause stem cell exhaustion, especially in the stem cell niche. When stem cells become exhausted, the capability of replacing senescent is lost, hence leading to a global organism aging (Lopez-Otin, Blasco et al. 2013). During normal aging process, the hallmarks comes in a specific order: first, the primary hallmarks appear, leading to a damage accumulation. Aiming to oppose the effects of the primary hallmarks, antagonist hallmarks comes to the picture. Being favourable at first, these hallmarks become negative overtime, as a consequence of the primary hallmark's exacerbation. At last, the integrative hallmarks appear when both primary and antagonist hallmarks have produced enough damage, compromising the homeostasis of the body. Together, these hallmarks are able to explain the aging process.

The role of hypothalamus in aging

The hypothalamus is a small part of the brain, duplicated symmetrically to each hemisphere, located below the thalamus, and surrounded by the optic chiasm, optic tracts and mammillary body. The hypothalamus is divided in 3 main parts: preoptic area, tuberal hypothalamus and posterior hypothalamus, each part containing several small nuclei. Every part contains a different type of neuron capable to communicate with each other. The preoptic area contains the ventrolateral preoptic nucleus, lateral preoptic area, medial preoptic area and suprachiasmatic nucleus and is responsible for sexual behaviour, circadian rhythm, wake-sleep cycle, electrolyte balance and thermoregulation. The tuberal hypothalamus includes lateral and anterior hypothalamic areas, dorsomedial, ventromedial, paraventricular, supraoptic and arcuate nuclei and is responsible for feeding behaviour, aggressiveness and autonomic and endocrine responses in general. The posterior hypothalamus contains mammillary bodies and posterior hypothalamic nuclei and is responsible for providing feedbacks to arousal system and hippocampus (fig. 2) (Saper and Lowell 2014).

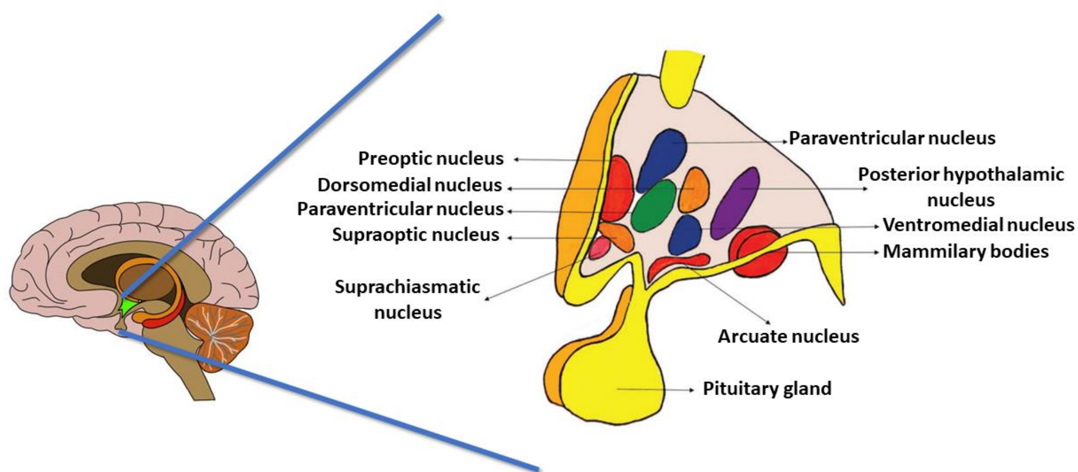


***Figure 2**- Schematic representation of a sagittal brain section and its hypothalamic nuclei: Preoptic nucleus, dorsomedial nucleus, paraventricular nucleus, supraoptic nucleus, suprachiasmatic nucleus, posterior hypothalamic nucleus, ventromedial nucleus, mammillary bodies and arcuate nucleus. (Pop, Crivii et al. 2018)*

The arcuate nucleus (ARC) is located in the lower part of the hypothalamus (Pearson and Placzek 2013), allowing this nucleus to communicate both with hypothalamus and extra-hypothalamic areas, namely with brainstem (Cone, Cowley et al. 2001). In this location, the blood brain barrier (BBB) is semi-permeable (Peruzzo, Pastor et al. 2000) allowing ARC to receive signals from circulatory nutrients and hormones (fig. 5). This strategic location makes the ARC the primary nutrient-sensing centre of the hypothalamus (Roh and Kim 2016). The ARC consists in 2 types of neurons: orexigenic neurons co-expressing neuropeptide Y (NPY) and agouti-related

protein (AgRP) (Broberger, Johansen et al. 1998), and anorexigenic neurons co-expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (table 1).

Being one of the most abundant peptide of the central nervous system (CNS), NPY is present all over the brain, namely in the ARC (Allen, Adrian et al. 1983). NPY acts via eight different NPY receptors (Y1-Y8) (Walther, Morl et al. 2011) being Y1, Y2 and Y5 the most abundant receptors in the body (Baraban 2004). With 36 amino acids (Tatemoto, Carlquist et al. 1982), NPY have a potent orexigenic effect, even in sated animals (Parrott, Heavens et al. 1986). As expected, inhibition of NPY decrease food intake and body weight (Shimokawa, Kumar et al. 2002) and anorexic mice showed a reduced NPY signalling (Broberger, Johansen et al. 1997). Controversial reports show however that mice overexpressing NPY did not increased food intake (Broberger, Johansen et al. 1997) and NPY knockout mice have a normal food intake and body weight (Erickson, Clegg et al. 1996), thus suggesting overlapping mechanisms of food intake regulation. NPY expression is regulated by energy-related factors (fig. 3), such as leptin, ghrelin, insulin, glucocorticoids and glucose, among others ((Minor, Chang et al. 2009), (Shimizu, Arima et al. 2008)). NPY is also involved in many homeostatic mechanisms, like adiposity, being also expressed in adipocytes (Kos, Harte et al. 2007). Apart from CNS, NPY is also present in sympathetic nerves, spleen and gut (Minor, Chang et al. 2009). NPY acts as a neurotransmitter, inhibiting adenylyl cyclase (Herzog, Hort et al. 1992) and increasing intracellular calcium levels (Jacques, Sader et al. 2000).

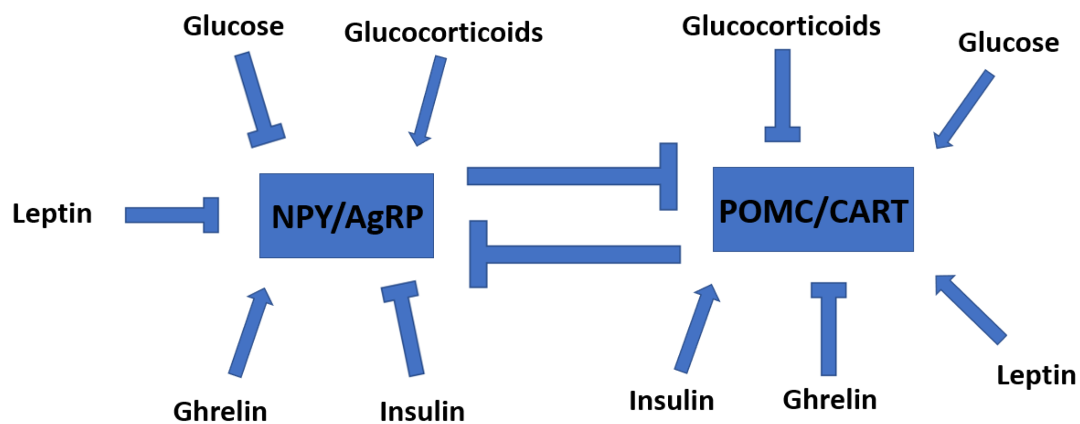


Figure 3 *- Regulation of NPY/AgRP and POMC/CART neurons by energy-related factors.*

AgRP neurons, along with NPY neurons, reduce energy consumption and increase appetite, which could result in weight gain (Shutter, Graham et al. 1997). More specifically, AgRP peptide blocks melanocortin receptor, thus inhibiting appetite control (Ollmann, Wilson et al. 1997). AgRP neurons are strongly regulated by ghrelin since their ablation lead to a lean phenotype, even in the presence of ghrelin (Bewick, Gardiner et al. 2005). By reverse, leptin negatively regulate AgRP, supressing is activity (Elmquist, Coppari et al. 2005). In comparison with NPY, AgRP orexigenic effect is longer since a single injection of AgRP in mice have an effect for more than a week (Hagan, Rushing et al. 2000).

POMC neurons are responsible for POMC peptide production, which is a precursor of several peptides such as melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH) by posttranscriptional processing. MSH binds to melanocortin receptors 3 (MC3R) and melanocortin receptor 4 (MC4R), triggering anorexigenic effects (Cowley, Pronchuk et al. 1999).

CART neurons are found all over the brain, and take part in several physiological processes such as memory, feeding, stress and anxiety, endocrine regulation, and drug abuse (Ahmadian-Moghadam, Sadat-Shirazi et al. 2018). In the ARC, CART neuropeptides are co-

expressed with POMC neuropeptides and, together, are able to inhibit food intake and body weight (Ahmadian-Moghadam, Sadat-Shirazi et al. 2018).
In a fed state, POMC/CART neurons are highly expressed while, in contrast, NPY/AgRP neurons have a lower expression. In a non-fed state, NPY/AgRP neurons are highly expressed while POMC/CART reach minimal expression. (Ziotopoulou, Mantzoros et al. 2000). Both ghrelin and GHSR-1a were found to be expressed in the ARC neurons, suggesting a close relationship between ghrelin and these neurons (Ferrini, Salio et al. 2009).

Table 1 *- Peptides expressed in the arcuate nucleus and their main functions.*

Peptide	Function
Neuropeptide Y (NPY)	Food intake and body weight induction (orexigenic effect)
agouti-related protein (AgRP)	Food intake and body weight induction (orexigenic effect)
pro-opiomelanocortin (POMC)	Food intake and body weight inhibition (anorexigenic effect)
cocaine- and amphetamine-regulated transcript (CART)	Food intake and body weight inhibition (anorexigenic effect)

As mentioned, the hypothalamus is a key component of the neuroendocrine system, which is known to have major roles in growth, development, reproduction, metabolism, systemic aging and, therefore, lifespan control. Its primary function is to link the nervous system to the endocrine system via pituitary gland, maintaining body homeostasis by detecting circulatory metabolites and hormones as well producing and releasing these hypothalamic hormones (table 2). Growth hormone (GH) and IGF-1 are two of these hypothalamic hormones. GH is responsible for stimulate growth and the production of IGF-1, which is responsible for systemic body growth. Both GH and IGF-1 have a higher concentration during the adolescence and gradually decrease during aging process (Kim and Choe 2019). Another important hormone produced and released by the hypothalamus is the gonadotropin-releasing hormone (GnRH). GnRH is released in portal vessel in a pulsatile manner and is responsible for the activation of gonadotropes in the pituitary, having a downstream effect in gonads (Maeda, Ohkura et al. 2010). This pulse generator is controlled upstream by kisspeptin neurons, neurokinin B and dynorphin A (together called KNDy neurons). Although the number of GnRH levels remains the same, KNDy neurons decrease with age, decreasing the pulse generation and consequently decreasing the release of GnRH leading to infertility both in male and female (Kunimura, Iwata et al. 2017). GnRH is also strongly negative regulated by IkB Kinase-β (IKK-β) and nuclear factor kB (NF-kB) (Zhang, Li et al. 2013).

Table 2 – Hypothalamic peptides and their main functions.

Peptide	Function	Concentration levels
Growth Hormone (GH)	Stimulates production of IGF-1	High levels during adolescence; gradually decrease during aging process
Insulin-Like Growth Factor 1 (IGF-1)	Systemic body growth	High levels during adolescence; gradually decrease during aging process
Kisspeptin	Positive Regulation of GnRH	High levels during adolescence; gradually decrease during aging process
Neurokinin B	Positive Regulation of GnRH	High levels during adolescence; gradually decrease during aging process
Dynorphin A	Positive Regulation of GnRH	High levels during adolescence; gradually decrease during aging process
Kinase-β (IKK-β)	Negative Regulation of GnRH	Varies
nuclear factor kB (NF-kB)	Negative Regulation of GnRH	Varies
Gonadotropin-releasing hormone (GnRH)	Activation of gonadotropes	Same levels during aging process

Other important mediator of aging is neural stem cells (NSC). NSC are located in a few brain regions being responsible for local neurogenesis (Lois and Alvarez-Buylla 1993), therefore correlated with age-related disorders (Encinas, Michurina et al. 2011). It was shown that by measuring NSC biomarkers, the hypothalamic NSC (htNSC) decrease with age leading to a neurogenesis decrease along aging process (Zhang, Kim et al. 2017). To prove it, they ablated htNSC and saw a cognitive decline and a decreased lifespan, which were reverted when they injected the mice with htNSC. They also reported exosomal miRNA as a partial modulator of anti-aging effect of htNSC. Investigators believe that most of hypothalamus age-related events are a consequence of microinflammation of the hypothalamus and these microinflammations are derived mostly from a unbalanced nutrients influx or autophagy malfunction (Tang, Purkayastha et al. 2015).

Ghrelin: gene and protein

Human ghrelin gene, GHRL, is located in chromosome 3, locus 3p25-26, and contains four coding exons and two non-coding exons, totalizing 511 base pairs from 5' to 3' (Ghrelin (Uniprot access entry: Q9UBU3) is a 28 amino acid protein, discovered in 1999 by (Kojima, Hosoda et al. 1999), being the only known peptide hormone, so far, having a post translational modification by a fatty acid.

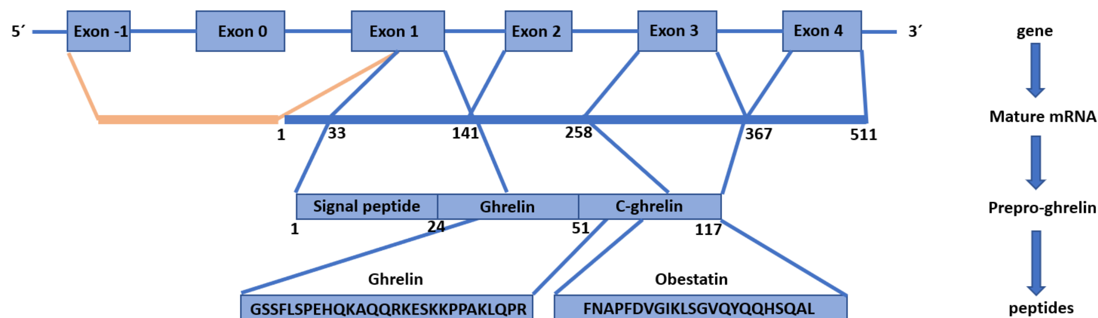


Figure 4 *- Ghrelin gene, mRNA and peptides.*

Exon 1 to exon 4 encodes for a peptide called preproghrelin composed by signal peptide, ghrelin itself and C-ghrelin, totalizing 117 amino acids (Kojima, Hosoda et al. 1999). Proghrelin is made of ghrelin (28 amino acids) and C-ghrelin (Korbonits, Goldstone et al. 2004). C-ghrelin (66 amino acids) is further cleaved into obestatin (23 amino acids), which is an hormone that, contrary to ghrelin, supress food intake (Zhang, Ren et al. 2005). Controversially, a significant number of studies cannot confirm obestatin anorexigenic effect (Hassouna, Zizzari et al. 2010), although it was found to be related with the pathogenesis of diabetes (Green and Grieve 2018). Signal peptidase is the enzyme responsible for the proteolytic cleavage in Arginine 23 (Arg23) of preproghrelin into signal peptide. Prohormone convertase 1/3 (PC 1/3) generates ghrelin by cleaving at Arginine 51 (Arg51) (Zhu, Cao et al. 2006). At this point, ghrelin is not active and to be so, it is required to suffer a post translational modification consisting in acylation by the hydroxyl group of the Serine 3 (Ser3) (Hosoda, Kojima et al. 2003). This activation is essential to allow ghrelin to cross the BBB and posterior bind to Growth Hormone Secretagogue Receptor (GHSR) (fig. 5) (Banks, Tschop et al. 2002). Contrary to acylation, ghrelin may also suffer de-acylation by the enzyme Acyl-Protein Thioesterase 1 (APT1) (Satou, Nishi et al. 2010) and by butyrylcholinesterase (BChE) (Chen, Gao et al. 2017).

Studies support that a high fat diet increase acylated ghrelin rather that unacylated ghrelin (Nishi, Mifune et al. 2012). In human blood plasma, the presence of desacyl ghrelin is higher than acyl ghrelin (Akamizu, Shinomiya et al. 2005) probably due to the low half-life time of acyl ghrelin.

Ghrelin is mainly produced by the stomach, in oxyntic cells (fig. 5), being responsible by eighty percent of circulating ghrelin but lower amounts can be produced by bowel, pancreas, pituitary gland, kidney and placenta (Hosoda, Kojima et al. 2006).

Ghrelin acylation is achieved by Ghrelin O-Acyl Transferase (GOAT) (fig. 5), discovered in 2008 by (Yang, Brown et al. 2008), belonging to Membrane O-Acyl Transferase (MBOAT) protein family and regulated upstream by leptin (Gonzalez, Vazquez et al. 2008). GOAT is found predominantly in stomach so the acylation may occur as soon as ghrelin is synthetized (Gonzalez, Vazquez et al. 2008).To demonstrate that GOAT is the only enzyme capable of perform ghrelin acylation, (Kirchner, Gutierrez et al. 2009) did a GOAT knockout mice and they were presented

with a total absence of acyl ghrelin. Recent reports shown that GOAT might be part of a nutrient sensing system, informing the body of the presence of nutrients (Kirchner, Gutierrez et al. 2009).

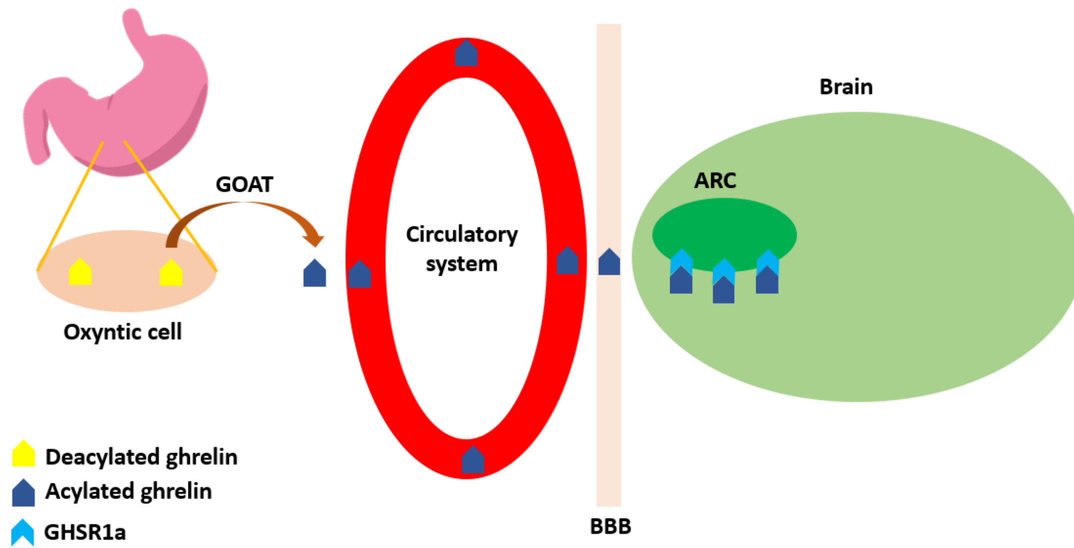


Figure 5 *- Ghrelin pathway: from production to the Brain. Deacylated ghrelin is produced in oxyntic cells, in stomach. GOAT catalyse ghrelin acylation by adding an octanoyl or decanoyl group in Ser3, allowing ghrelin to pass from the circulatory system to brain through blood brain barrier. In the brain, acylated ghrelin is able to bound to GHSR-1a receptor and trigger signalling pathways.*

Growth Hormone Secretagogue Receptor

Ghrelin has a natural receptor called growth hormone secretagogue receptor (GHSR), which is a G-protein coupled receptor (GPCR) with seven transmembrane domains. It is primarily responsible to stimulate the release of hormones (Table 3) like the ACTH, cortisol, prolactin and GH, which is a peptide hormone secreted in a pulsatile manner from somatotroph cells of the anterior pituitary gland and is responsible for the release of IGF-1 (Nicholls and Holt 2016).

Table 3 *– Peptides released by GHSR-1a stimulation.*

Peptide	Function
Growth Hormone Secretagogue Receptor (GHSR)	Ghrelin's receptor / Hormones releasing
Adrenocorticotropic Hormone (ACTH)	Stimulates production and releasing of cortisol
Cortisol	Stress response
Prolactin	Stimulates milk production
Growth Hormone (GH)	Stimulates production of IGF-1
Insulin-Like Growth Factor-1 (IGF-1)	Systemic body growth

GHSR1 gene is located on chromosome 3, locus 3q26.2 ((McKee, Palyha et al. 1997), (Colinet, Vanderick et al. 2009)) being composed of 2 exons and 1 intron. GHSR1 gene encodes for GHSR-1a protein, a 366 amino acid long functional form of GHSR that is constitutively active. This gene also encodes for GHSR-1b, which is a non-spliced and non-functional variant with 5 transmembrane domains (Gnanapavan, Kola et al. 2002). More specifically, the regions between N-terminal and the 5th transmembrane domains of GHSR-1a arise from exon 1 while exon 2 gives rise the rest of the protein. GHSR-1b, at is turn, emerge from exon 1 (McKee, Palyha et al. 1997) (fig. 6).

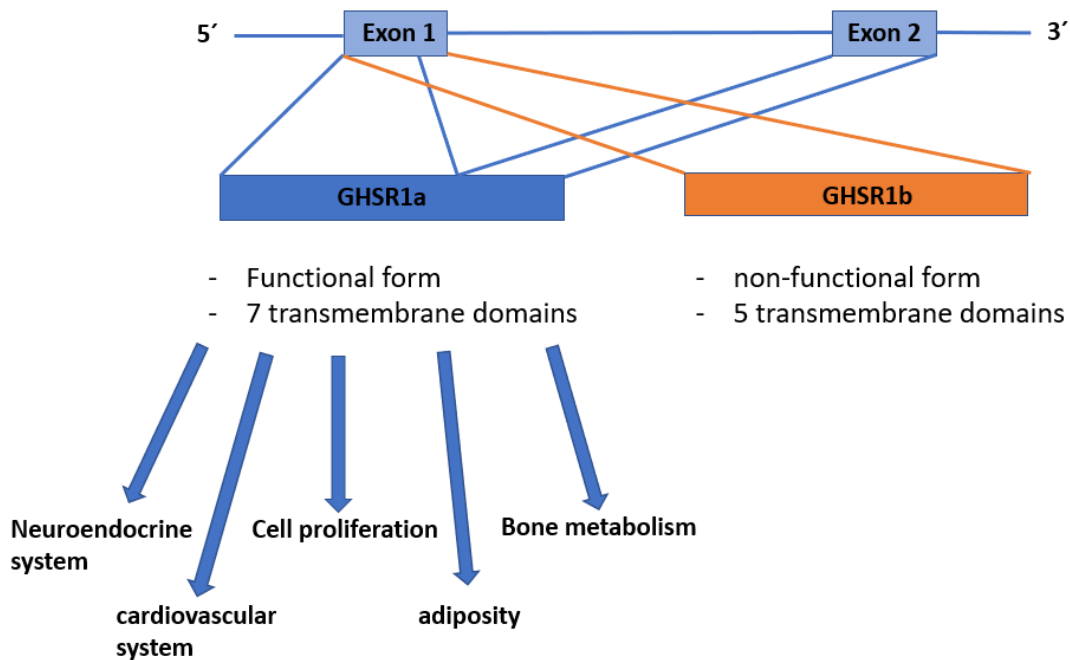


Figure 6 *- Growth Hormone Secretagogue Receptor (GHSR) gene, resulting peptides and respective functions.*

Although no specific role of GHSR-1b is known at this time, GHSR-1b seems to spread all over the body (Gnanapavan, Kola et al. 2002). GHSR1 gene expression was found in countless types of cancers, suggesting a role in regulation of cell proliferation (Jeffery, Herington et al. 2003). GHSR-1a were discovered, as well, in cardiovascular system, namely in myocardium and aorta artery (Gnanapavan, Kola et al. 2002). Additionally, GHSR-1a plays an important role in bone metabolism. This is supported by other findings showing that when proliferative effect of ghrelin on osteoblasts was attenuated in the presence of GHSR-1a antagonist (Kim, Her et al. 2005). Controversially, (Gnanapavan, Kola et al. 2002) did not found GHSR-1a mRNA expression in bone. Constitutive activity seems to be required for normal development of human body, maintaining basal levels of GH (Pantel, Legendre et al. 2006). In fact, GHSR-1a knockout mice fails to stimulate GH secretion and appetite induction after ghrelin administration (Sun, Wang et al. 2004). Despite being constitutively active, GHSR-1a requires a stable bound with ghrelin to change its conformation and be able to interact with other G proteins. Namely, the binding of ghrelin promotes the release of guanosine diphosphate (GDP) from the receptor and binding, instead, guanosine triphosphate (GTP), thus initiating signalling cascades (Camina 2006). One of this signalling pathways is in NPY-containing neurons. GHSR-1a mediates calcium influx when the receptor is activate by ghrelin binding (Kohno, Gao et al. 2003). The bound between ghrelin and his receptor can be reversed by a process called desensitization, which is a combination of the uncoupling of the receptor from the active form and the internalization to intracellular compartments (Yin, Li et al. 2014). Hence, this internalization suggests that GHSR-1a has the

qualification to mediate ghrelin uptake and degradation. GHSR-1a can be found in highest concentrations in the pituitary gland and hypothalamus, namely in the ARC. In fact, the presence of GHSR-1a in the ARC is so important that mice lacking the receptor in ARC decreased GH secretion, adipose tissue and body weight (Shuto, Shibasaki et al. 2002). GHSR-1a expression in the ARC can be reduced by the presence of leptin, an anorexigenic hormone secreted by white adipose tissue (WAT), suggesting ghrelin/leptin system as the major regulator of GHSR-1a expression (Zigman, Jones et al. 2006) . Recent reports also shown that 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) have is activity in hypothalamus mediated by ghrelin and leptin (Andersson, Filipsson et al. 2004). Lower concentrations are detected in pancreatic islets, adrenal glands, thyroid, myocardium (Guan, Yu et al. 1997) and sacral, lumbar and thoracic regions of the spinal cord (Ferens, Yin et al. 2010). In the brain, GHSR-1a is expressed in hypothalamus, cortex, *substantia nigra*, pons and medulla oblongata (Mani, Walker et al. 2014). GHSR-1a has been predicted to function as a homodimer (Holst, Brandt et al. 2005), but a heterodimer of GHSR-1a and GHSR-1b is necessary to the complex inhibition preventing ghrelin from binding (Leung, Chow et al. 2007). Heterodimerization can also occur with MC3R (Rediger, Piechowski et al. 2011), probable G-protein coupled receptor 83 (GPR83) (Muller, Muller et al. 2013), dopamine receptor 2 (Kern, Albarran-Zeckler et al. 2012) and serotonin receptor 2c (Schellekens, van Oeffelen et al. 2013), all inhibiting ghrelin action.

Ghrelin role in physiological functions

Food Intake

When ghrelin was discovered, the authors described this particularly peptide as the "Hunger Hormone", because of the major role that ghrelin plays in food intake (fig. 7). For example, ghrelin suppression after food intake is proportional to the quantity of ingested food (Callahan, Cummings et al. 2004).

Despite not knowing the existence of ghrelin at the time, (Willesen, Kristensen et al. 1999) suggested that the endogenous ligand that activates GHSR-1a plays a major role in hunger, and, therefore, food intake. They also discovered that, in the ARC, 95% of the NPY-expressing cells also express GHSR-1a and only 8% of POMC neurons express GHSR-1a. Both NPY and AgRP are so important for food intake induction by ghrelin that mice lacking both NPY and AgRP fails to initiate food consumption (Arvat, Maccario et al. 2001).

Although ghrelin is commonly known as "Hunger hormone", high levels of ghrelin does not necessarily mean obesity, nor low levels mean anorexia. In fact, Shiiya and colleagues (Shiiya, Nakazato et al. 2002) did an elegant cohort study showing that ghrelin levels are higher in patients with anorexia nervosa and lower in patients with common obesity. These findings suggest that, despite ghrelin playing an important role in food intake, cannot induce food intake only by itself.

Studies shown that ghrelin IV injection induce GH release (Arvat, Di Vito et al. 2000) as well ACTH and prolactin, both hormones related to growth (Arvat, Maccario et al. 2001). To achieve maximal GH release, ghrelin can also signal from the stomach to the brain via vagus nerve (Date, Murakami et al. 2002).

Ghrelin can also induce appetite by interacting with dopamine system (Abizaid, Liu et al. 2006), opioids system (Romero-Pico, Vazquez et al. 2013), cannabinoids system (Kola, Farkas et al. 2008) and serotonin system (Nonogaki, Ohashi-Nozue et al. 2006).

Acylated version of ghrelin plays an important in AMPK signalling pathways. AMPK activation causes inhibition of acetyl-CoA carboxylase (ACC) (Andersson, Filipsson et al. 2004) increasing mitochondrial metabolism (Minokoshi, Kim et al. 2002), production of ROS and

stimulation of uncoupling protein 2 (UCP2) and finally increases the expression of orexigenic neuropeptides (Andrews, Liu et al. 2008).

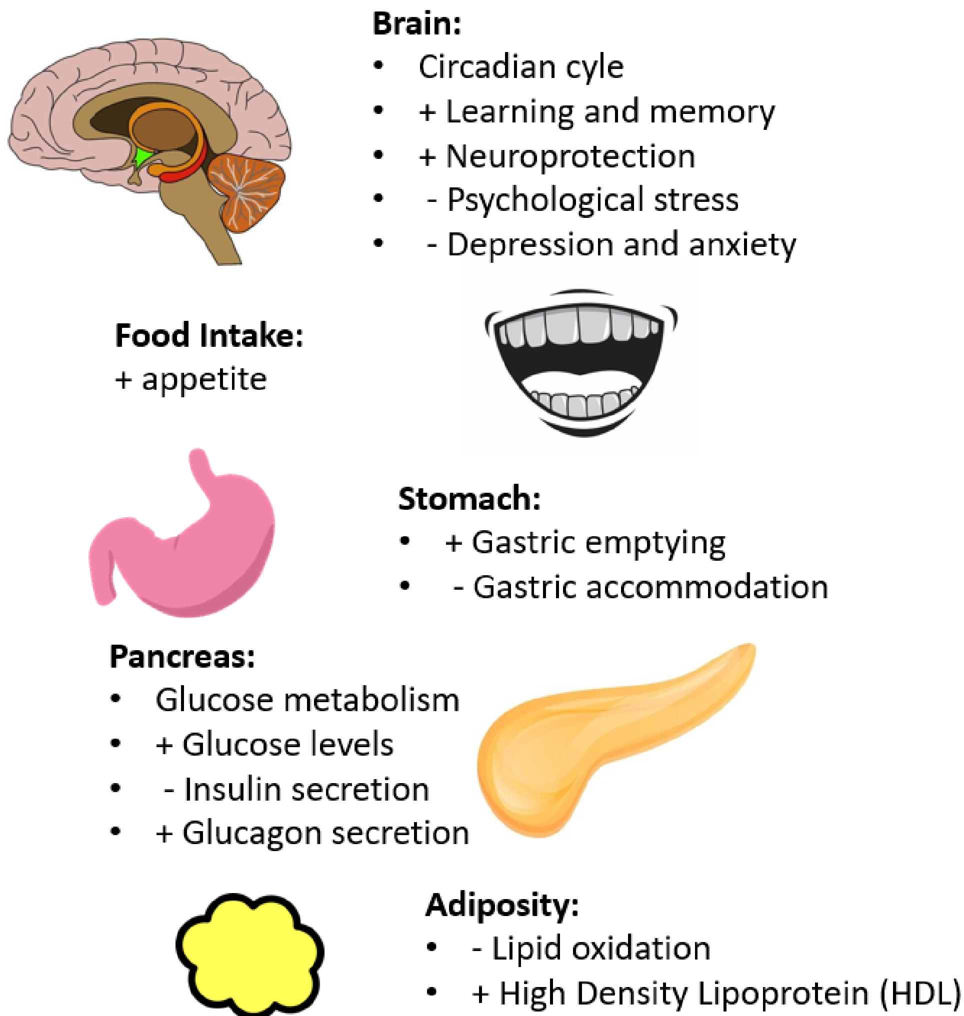


Figure 7 *- Ghrelin role in physiological functions.*

Glucose metabolism

The fact that GHSR-1a appears in pancreatic islets shows the importance of ghrelin in glucose metabolism. A study showed that acute administration of acylated ghrelin increase glucose levels and decrease insulin secretion, suggesting a non GH-mediated hyperglycemic effect (Broglio, Arvat et al. 2001). Another study corroborate this fact by doing a GHSR-1a rescue of a knockout mice and successfully restore glucose blood level to normal value (Wang, Liu et al. 2014). The negative correlation between insulin and ghrelin seems to be regulated by a non-canonical GHSR-1a signalling pathway (Dezaki, Kakei et al. 2007). Ghrelin can also have a role in glucagon system. A study found an abundant expression of GHSR-1a in glucagon-producing α-cells and suggested the ability of ghrelin to engage this cells inducing glucagon secretion (Chuang, Sakata et al. 2011).

Adiposity

When it comes to adiposity and lipid metabolism, ghrelin can induce fatty acid accumulation (Tschop, Smiley et al. 2000). Sympathetic nervous system seems to be able to mediate fatty acid storage and, by reverse, inhibit fatty acid oxidation, when ghrelin is administrated in the body (Theander-Carrillo, Wiedmer et al. 2006). More specifically, continuous administration of ghrelin seems to increase HDL cholesterol levels in blood plasma (Perez-Tilve, Hofmann et al. 2010), compelling ghrelin signalling pathway to a therapeutic target in disorders like dyslipidemia. Despite that, GHSR-1a seems to play a more important role in adiposity than ghrelin itself. For example, ablation of ghrelin producing cells in adult mice failed to affect food intake or body weight (McFarlane, Brown et al. 2014), but mice lacking GHSR-1a have a reduced body weight and adiposity (Zigman, Nakano et al. 2005). Plus, ablation of GHSR-1a in neurons shown a reduction on adiposity by enhancing thermogenesis in brown adipose tissue (BAT) (Lee, Lin et al. 2016). In liver, ghrelin deletion prevents hepatic steatosis through downregulation of p300/C-EBPα/DGAT1 pathway and, subsequently, decreased liver weight (Guillory, Jawanmardi et al. 2018). Together, these studies suggest that adiposity is regulated by energy consumption but not food intake.

Ghrelin in the brain

The importance of ghrelin in the brain is supported by findings showing that ghrelin expression is increased in stomach in a fasting state but not in pituitary or hypothalamus, suggesting that ghrelin plays a role in the brain not only related to food intake (Torsello, Scibona et al. 2003). Innumerable evidence supports the importance of ghrelin in the hippocampus in terms of learning and memory, where a single dose of ghrelin enhances episodic memory in mice (Carlini, Varas et al. 2004) and synapse formation, improving spatial memory (Diano, Farr et al. 2006).

Neuroprotective effects of ghrelin were first shown in a study, where they demonstrated that a continuous administration of growth hormone releasing peptide-6 (GHRP-6) (synthetic ligand for GHSR-1a) increased expression of proteins related to neuroprotection (Frago, Paneda et al. 2002). After, it was shown the ability of GHRP-6 to inhibit caspases 3 and 9 and, therefore, prevent cell death (Paneda, Arroba et al. 2003).

In addition, several studies have shown a potential role of ghrelin in different neurodegenerative diseases. In Parkinson's Disease (PD), ghrelin has a neuroprotective effect by enhancing dopamine availability during neurodegeneration and increasing mitochondrial respiration, making the neurons more resilient. This effect might be due to the decreasing of ROS (Andrews, Erion et al. 2009). Ghrelin can also increase AMPK in the hypothalamus, promoting neuronal survival but the exact mechanism remains unclear (Andrews, Liu et al. 2008). Other way that ghrelin may confer neuroprotection is by inhibiting mTOR activity (Dong, Wang et al. 2015).

Huntington's Disease (HD) is characterized by continuous involuntary movements, cognitive and psychiatric symptoms and autonomic disfunction due to a mutation in huntingtin gene (Landles and Bates 2004). In the early stages of disease, caspases genes become upregulated ((Ona, Li et al. 1999), (Chen, Ona et al. 2000)), and ghrelin can inhibit the pathways that comes from the activation of these genes, thus preventing neuronal apoptosis (Zhang, Yang et al. 2013).

Ghrelin also has the ability to increase cAMP response element binding protein (CREB) promoting brain derived neurotrophic factor (BDNF) (Ma, Zhang et al. 2011).

In terms of adult hippocampal neurogenesis (AHN), ghrelin plays an important role on is mediation having a proliferative effect on hippocampal progenitor cells, increasing AHN

(Johansson, Destefanis et al. 2008). This makes ghrelin a potential therapeutic compound in neurodegenerative Disease.

Other functions

Recent reports described a variety of roles of ghrelin in distinct areas like cardiovascular system, learning and memory, psychological stress, mood and anxiety, depression, thymopoiesis, circadian cycle and reward system (Muller, Nogueiras et al. 2015).

Expression of ghrelin and GHSR-1a mRNAs were found in both human and mice cardiomyocytes, suggesting a role in cardiovascular system (Iglesias, Pineiro et al. 2004). Evidences of ghrelin production by vascular endothelial cell were also found (Kleinz, Maguire et al. 2006). Ghrelin has the ability to improve cardiovascular function like cardiac index and stroke volume index ((Nagaya, Kojima et al. 2001), (Tritos, Kissinger et al. 2004)). Furthermore, dysregulation of plasma ghrelin levels was associated with coronary artery disease, namely hypertension (Poykko, Kellokoski et al. 2003). Also, patients were found to have lower ghrelin levels right after myocardial infarction (Matsumoto, Yasuda et al. 2013). All these studies suggested ghrelin as potential therapeutic target for heart-related diseases.

Several studies reported a correlation between ghrelin and bone. *In vitro* experiments, showed a beneficial effect of ghrelin on osteoblast proliferation and differentiation and apoptosis inhibition (Nikolopoulos, Theocharis et al. 2010). In order to induce proliferation, ghrelin acts both in mitogen activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways ((Nanzer, Khalaf et al. 2004), (Baldanzi, Filigheddu et al. 2002)). This effect is transversal for most cell types. A study proposed an autocrine/paracrine regulation, considering the discovery of ghrelin mRNA expression in bone biopsies (Delhanty, van der Eerden et al. 2006).

Circadian rhythm is generated in the hypothalamic suprachiasmatic nucleus and is primarily affected by light and integrated by visual pathways, allowing a geophysical time location (Moore 1983). Release of ghrelin is somehow related to sleep/wake cycle since short sleep individuals (5 hours sleep per night) have 15% more circulating ghrelin than normal sleep individuals (Taheri, Lin et al. 2004). Controversial, men suffering from chronic insomnia were found to have lower levels of circulating ghrelin comparing with men with normal sleep behaver (Motivala, Tomiyama et al. 2009). It is yet to discovered if poor sleep induces ghrelin release or a deregulated ghrelin release impel poor sleep. When it comes to hunger, a study has shown that acylated ghrelin varies in a circadian manner, even on fasting conditions (Qian, Morris et al. 2019). This circadian variation explains why individuals are able to skip breakfast after 8 hours fasting, but during the day the same individuals are hungry 3 hours after dinner. The same experiment also shown that circadian misalignment increases postprandial appetites, once again suggesting ghrelin release in a circadian manner.

Regulation of ghrelin secretion

Regarding food intake, it is now well established that ghrelin levels increase in a pre-prandial state and decrease to baseline in a postprandial state (Cummings, Purnell et al. 2001). However, the exact mechanisms that lead to ghrelin biosynthesis and releasing is yet to be clear. Nevertheless, studies suggest that these mechanisms are triggered by key compounds like glucose and not by physical phenomena in stomach, as filling the stomach of a fasting mouse with glucose decrease ghrelin levels, however, filling with water produces no changes, suggesting a post gastric regulation (Tschop, Smiley et al. 2000). Some reports suggested that concentrations of ghrelin and leptin in blood plasma are inversely proportional and when ghrelin concentration is high and, by contrast, leptin is low, a powerful orexigenic effect happen

(Bagnasco, Kalra et al. 2002). It was also shown that both acyl and desacyl ghrelin can be lowered (even in a long term food deprivation where is well established that ghrelin levels in plasma increase), when the mice are treated with reserpine, a drug that depletes adrenergic neurotransmitters from sympathetic neurons, suggesting a key role of these neurons in ghrelin secretion (Zhao, Sakata et al. 2010). Moreover, it was found that ghrelin secretion increases in the cephalic phase of digestion, which is regulated by hypothalamus, suggesting his importance in regulation of ghrelin secretion (Monteleone, Serritella et al. 2008). In fact, a study with the injection of several candidate compounds into gastric mucosa, found out that epinephrine, norepinephrine, endothelin and secretin can induce ghrelin release, whereas gastric hormones like somatostatin and gastrin do the opposite, suggesting that ghrelin is positively regulated by the sympathetic nervous system and negatively regulated by gastric system (de la Cour, Norlen et al. 2007). Ghrelin secretion itself are regulated mainly by GPCR (Engelstoft, Park et al. 2013).

Possible effects of desacyl ghrelin

The majority of circulation ghrelin is, in fact, desacyl ghrelin, with a normal ratio of acyl ghrelin of 1:55 (Hosoda, Kojima et al. 2000), although it varies, depending on metabolism and period of the circadian rhythm. Desacyl ghrelin does not bind to GHSR-1a due to the lack of the fatty acid but that does not mean exactly the lack of function. Despite not knowing at this point a specific function for desacyl ghrelin, evidence supports that it might play a role in insulin releasing independently of GHSR-1 mediated pathways (Gauna, Delhanty et al. 2006), regulation of body temperature (Heppner, Piechowski et al. 2014), muscle atrophy (Porporato, Filigheddu et al. 2013) and lipid metabolism (Thompson, Gill et al. 2004). In human breast cancer (Cassoni, Papotti et al. 2001) and prostate cancer (Cassoni, Ghe et al. 2004), desacyl ghrelin can inhibits tumor progression, even in cells that not express GHSR-1a.

It is also possible that ghrelin might inhibit the orexigenic effect of acyl ghrelin due to the interaction between the 2 peptides (Inhoff, Monnikes et al. 2008). Plus, transgenic mice overexpressing desacyl ghrelin had a reduced body size, reduced body weight (Ariyasu, Takaya et al. 2005) and an increased insulin sensitive (Zhang, Chai et al. 2008), suggesting a role of desacyl in glucose homeostasis.

Objective

Increased lifespan leads to an augmented risk of age-related pathologies like cancer, diabetes, cardiovascular disorders and neurodegenerative diseases (Lopez-Otin, Blasco et al. 2013). Treating these disorders must be a priority to allow a quality and proper aging process. Ghrelin signalling pathways were shown to be present in several systems, highlighting ghrelin as an important player in whole-body metabolism (Muller e Nogueira 2015). The goal of this project is to evaluate whole-body effect of subcutaneous injection of ghrelin in aged mice. More specifically, evaluate the effect of ghrelin administration in food intake, body weight, organs function and animal behaviour.

Our hypothesis is that administration of ghrelin in mice improves aging hallmarks.

This investigation could be a starting point for a new approach in the prevention of several age-related conditions.

Material and Methods

In Vivo experiments

Animals

For the purpose of this study, in-house breed animals were used, both male and female C57BL/6J (founders bought from Charles River, Barcelona). Animals were kept in the animal pathogen-free facility of Centre for Biomedical Research (CBMR) of the University of Algarve. Animals were divided in two different cohorts (fig. 8). In cohort 1, we had 8 animals, 17-month-old at the beginning of the trial and 18 months at the end of the trial, weighing 28-38g. In cohort 2, we had 22 animals, 16-month-old at the beginning of the trial and 18-month-old at the end of the trial, weighing 24-39g. Cohort 1 lasted for 1 month (30 days) while cohort 2 lasted for 2 months (60 days). In each cohort, mice were equally divided in 2 different groups: Control and experimental. Mice were kept at 22±2ºC room temperature, 12-hour light-dark cycle, fed on a standard laboratory diet and autoclaved water *ad libitum*. Food and body weight were measured once a week. Body weight gain was calculated as a % of the initial body weight (day 1) and food intake was calculated by the ratio of ingested food (g) per total body weight for cage (g) and multiplied by body weight of each mouse [(ingested food/total body weight for cage) X body weight of each mouse)]. Organ weight were normalized with total animal body weight. Mice were also group-housed by gender in cages and paper nesting material was provided (see annex 1). All procedures were made under the premise of minimize pain and stress to the animals, in conformity with the European Union Directive (86/609/EEC). To the researchers involved in mice manipulation, had been given appropriate training and certification from *Direção Geral de Alimentação e Veterinária (DGAV),* according to Federation of Laboratory Animal Science Associations (FELASA). No unnecessary procedures were performed on mice. The project and animal procedures were licensed by DGAV (ALBUM project, authorization 421/2019).

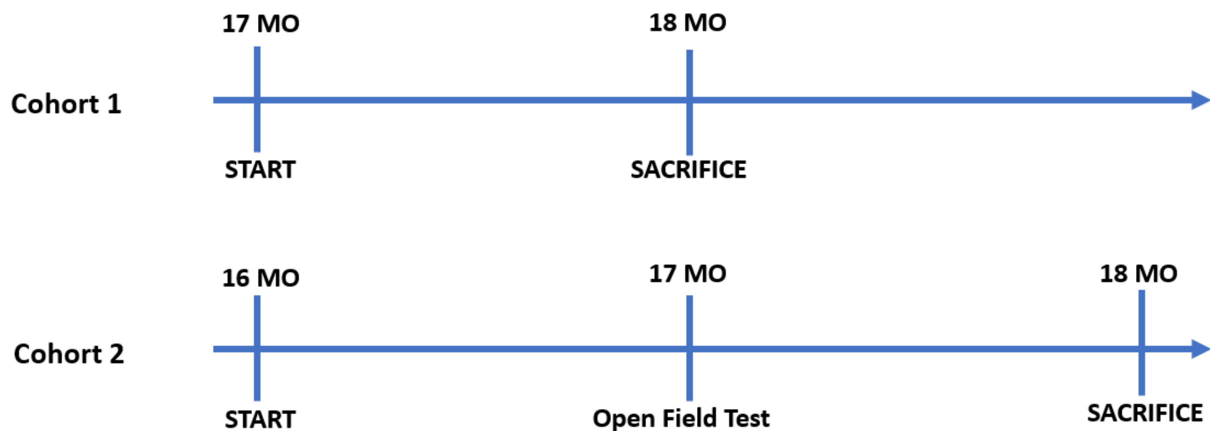


Figure 8 *- Experimental design: Cohort 1 started with 17-month-old mice and ended 1 month later (18-month-old mice). Cohort 2 started with 16-month-old mice and ended 2 month later (18-month-old mice). In the middle of experiment, mice were submitted to an open field test.*

Ghrelin administration

In order to evaluate the effects of ghrelin, mice were randomly divided in two different groups (Table 4): subcutaneous administration of sodium chloride (0.9% NaCl) and subcutaneous administration of ghrelin. In cohort 1 animals were injected 6-days a week for one month, with 8 animals (*n=8*) divided equally in both groups (*n=4* for NaCl and *n=4* for ghrelin). In cohort 2, animals were injected 6-days a week for two months, with 22 animals (*n=22*) divided equally in both groups (*n=9* for NaCl and *n=13* for ghrelin).

Table 4 *– Scheme of cohorts. Mice were randomly divided according to gender.*

	1st Cohort		2nd Cohort	
	NaCl	**Ghrelin**	**NaCl**	**Ghrelin**
Male	2	3	4	7
Female	2	1	5	6

Mice were injected subcutaneous according to (Shimizu 2004) everyday, except Sundays, at 9am. Injection dose was 50µg of ghrelin per kilogram of mice bodyweight. Ghrelin was diluted in saline solution (0.9% NaCl) at 1.5µg/µl. Injection volume was adapted according to body weight (3.33µl/g of bodyweight). The same calculus for injection volume was applied to control mice.

Tissues and blood collection

At the end of the experiment of each cohort, animals were coma induced by isoflurane in an anesthesia chamber and, immediately after that, mice were sacrificed by decapitation. All sacrifice took place during light phase of the diurnal cycle.

Aiming to perform histological processing, the following tissues were collected: liver, heart, thymus, spleen, descendent aorta, pancreas, stomach, WAT, BAT, dorsal skin, right back leg muscle, left leg, gut and testicles/ovaries. Samples were collected to 15ml tube with % neutral buffered formaldehyde solution (PanReac Applichem ITW Reagents) (ratio tissue/formalin of 1:20) and storage at room temperature until further analysis. To perform RNA analyses (gene expression by qRT-PCR) and protein analysis (Western-Blot), the following tissues were collected: brain, hypothalamus, liver, WAT, BAT, dorsal skin and right back leg muscle. Spleen, WAT and Liver were weighed with an analytical balance (AA-200 by Denver Instrument Company) after collection. Tissues were collected to a 2ml tube and storage at -80ºC until further analysis. Blood was also collected to a 2ml tube and serum was obtained by centrifugation (2000g for 15min at 4ºC) and stored at -20ºC until further analysis.

Behavior tests

Open Field test

Open field tests were performed for cohort 2, 1 month after beginning of experiment. Tests were conducted during light phase of diurnal cycle in a white light illuminated and sound-attenuating room. Open field tests were conducted in a 40x40x40cm transparent arena (without roof). Arena's floor was divided into nine equally squares. Mice were placed in the center of the arena and explored freely for 10min. Movement activity was recorded by video using a GoPro Hero camera (GoPro Inc., USA). Between tests, arena was cleaned with 70% ethanol (v/v). Total

distance, mean speed, time spend immobile, immobile episodes, number of line crossing, number and time of rearing, number and time grooming and number of entries and time in the middle were the measured parameters using the ANY-maze behavioral tracking software (Stoelting Company, Europe). Number of rearing (both front paws leaned against the wall) and grooming were counted as vertical activity while the number of crossing lines were counted as horizontal activity. By performing Open Field test, we aim to measure anxiety levels in mice.

Analysis

Histology

In order to perform tissue processing and paraffin inclusion, tissues were placed in specific processing cassettes (Labor Spirit). Dehydration occurred with 70% ethanol (v/v) for 1 hour, followed by 95% ethanol (v/v) for 45min, 95% ethanol (v/v) for 40min and two series of 100% ethanol (v/v) for 1 hour. Dehydration process was followed by clearing with two series of xylene (Fisher Chemical) for 1 hour and infiltration with two series of paraffin (Luso Palex) in the incubator at 56ºC for 1 hour. Tissues were then mounted in embedding molds (Tebu-bio) and filled with liquid paraffin at 56ºC. Cassettes were placed on the block and cooled down to room temperature. Blocks were sectioned using a HM 325 Rotary Microtome (Thermo Fisher Scientific) at room temperature. Blocks containing WAT or BAT had a final thickness of 4 or 5 µm while other organs or tissues were sectioned at 3 or 4 µm of thickness. All sections were placed in microscopy slides.

Hematoxylin-eosin staining was performed according to the "Eosin Y-solution 0.5% aqueous" protocol (Merck Milipore) followed by "Hematoxylin solution modified acc. to Gill II" (Merck Milipore). After drying, sections were mounted with Richard-Allan Scientific Mounting Medium (HM325, Thermo Fisher Scientific) and covered with microscopy slide slips.

Images from sections were obtained using an Axio Imager Z2 microscope (Carl Zeiss) and analyzed with Image J – Fiji software.

Protein analyses

In order to measure both total and specific protein present in the collected tissues, protein was extracted with QIAzol™ Lysis Reagent (QIAGEN). Samples were mechanically homogenized with a syringe (Terumo) and 23G needle (BD Microlance™), sited for 5min and 200 µl of d-chloroform (VWR Chemicals) were added to the homogenate. Samples were then centrifuge at 12000rpm for 15min at 4ºC (GYROZEN 1730R). Aqueous phase (top layer with approximately 500 µl) was transferred to a RNAse free tube and kept for posterior RNA analyses. Next, 300µl of 100% ethanol (v/v) was added to the interphase and phenol phase, incubated at room temperature for 3min and then centrifuged at 2000g for 2min at 4ºC. The supernatant (protein fraction) was transferred to a 15ml tube and 1,5ml of isopropanol (Fisher Chemical) was added aiming to precipitate protein. Samples were incubated at room temperature for 10min. A centrifugation was performed at 12000g for 10min at 4ºC and the supernatant was discarded. 1ml of guanidine-ethanol solution (SIGMA) was added and, after incubation at room temperature for 20min, samples were centrifuged at 7500g for 5min at room temperature. Supernatant was again removed. The process of adding guanidine-ethanol and centrifuge was repeated 2 more times. 1ml of 100% ethanol (v/v) was added to the pellet, followed by incubation at room temperature for 20min. Samples were centrifuged at 7500g for 5min at room temperature and supernatant discarded. Pellet was air-dried. 100 µl Urea (SIGMA)/dithiothreitol (DTT) solution (Fisher Scientific) was added to the samples followed by 2 incubations: one at

room temperature for 1 hour and the other at 95ºC for 3min. Samples were sonicated using Diagenode's Bioruptor® Pico, following manufacturer's instructions.

Total protein was measured using Bradford assay (NZY Tech), having Bovine Serum Albumin (BSA) as standard. To perform the standard curve, the following concentrations of BSA were used: 2000µg/ml, 1000µg/ml, 800µg/ml, 600µg/ml, 300µg/ml, 150µg/ml and 0µg/ml. Bradford assay was performed in a Falcon ® 96 Well Clear Microplate. Samples were diluted 20 times in H_2O MilliQ and 5µl were loaded in the plate. 5µl of standards were also loaded. 250µl of Bradford reagent were loaded in all wells and the plate was incubated at room temperature for 10min. Absorbance was read in the Glomax Multi Detection System (Promega) luminometer in a wavelength of 600nm.

Western-Blot

In order to identify specific proteins, present in the tissues, Western-Blot (WB) technique was performed.

To perform electrophoresis, Sodium Dodecyl Sulfate-polyacrylamide (SDS-PAGE) gels were used. The resolving gel, at 12%, was prepared using the following compounds: 3.65ml of H_20 milliQ, 2.8ml of resolving buffer [Tris HCl 1.5M, pH=8.3 and Sodium Dodecyl Sulfate (SDS) (Fisher Chemical) at 4%], 4.59ml of 30% acrylamide-Bis (BIO-RAD), 40µl of 10% Ammonium Persulfate (APS) (Fisher Chemical) and 9.2µl of tetramethyl-ethylene-diamine (TEMED) (Fisher Chemical). The stacking gel, at 4%, was prepared using the following compounds: 175ml of H_2O milliQ, 1.25ml of stacking buffer [Tris HCl 0.5M, pH=6.8 and SDS at 0.4%], 0.5ml of 30% acrylamide, 50µl of 10% APS and 5µl of TEMED. Electrophoresis buffer was Tris-base (Fisher Chemical), Bicine (PanReac AppliChem) [25mM Tris; 25mM Bicine; 1%(w/v) SDS; pH 8.3]. The amount of loaded protein in each lane was 60µg/20-30µl for samples and 8µl of protein marker (NZY Tech). Electrophoresis was performed for 30min at 80V and for 1 hour at 120V. After electrophoresis, it was necessary to perform membrane activation. For that, polyvinylidene fluoride membranes (PVDF) (Merck Millipore) were placed in 99.9% methanol (Fisher Chemical) for 20sec, washed with distilled H_2O for 5min, and then electrophoresis buffer [CAPS (Fisher Chemical) with 10% methanol] for 15min. After membrane activation, electrophoretic transfer was performed in a Wet Tank Blotting System (BIO-RAD) at 500 mA for 4 hours at 4ºC. Membranes were then blocked with 30ml of a 5% BSA (NZY Tech) blocking solution in TBS (Tris-base, NaCl and H_2O) and Tween™ 20 (Fisher BioReagents™) (TBS-T: Tris-Buffered Saline-Tween) for 1 hour at room temperature.

Table 5 *- Antibodies used in Western-Blot analysis.*

	Antibody	Host	Dilution	Molecular weight (KDa)
Primary	Anti-Interleukin 6 (proteintech®;)	Mouse	(1:1000)	24
	Anti-Agouti-Related Peptide (Phoenix Pharmaceuticals, INC.)	Mouse	(1:500)	14
	Anti-Proopiomelanocortin (Phoenix Pharmaceutical, INC.)	Rabbit	(1:500)	29
	Anti-β-Actin (SIGMA)	Mouse	(1:500)	42
Secondary	Anti-Mouse (GE Healthcare)		(1:10000)	
	Anti-Rabbit		(1:10000)	

PVDF membranes were incubated with primary antibody (see table 5), diluted in a 5% BSA and sodium azide (Alfa Aesar – 99%) solution. The incubation took place overnight at 4ºC with shaking. After the incubation, membranes were then washed three times with TBS-T 1X for

10min (each time) and incubated with specific secondary antibody (see table 5), diluted in 5% BSA for 2 hours, with shaking. Membranes were washed again tree times with TBS 1X for 10min (each time) and then incubated for 5min with 600µl of Amersham™ ECL™, Prime or Select, Western Blotting Detection Reagent (GE Healthcare – Life Sciences) in light protected conditions. Proteins were detected by chemiluminescence on a ChemiDoc™ XRS$^+$ Imaging System (BIO-RAD) coupled to Image Lab Software (BIO-RAD). Membrane re-probing was performed by washing membranes three times with TBS-T 1X (1 time for 10min, 1 time for 30min and 1 time for 1 hour each). Membranes were then incubated with the respective antibodies. In order to remove both primary and secondary antibodies from the membrane, stripping technique was performed. Membrane was washed with 40% methanol for 30min, distilled H_2O for 5min, then 0.2M NaOH for 5min and distilled H_2O for another 5min. Membrane were placed in BSA 5% blocking solution for 1 hour at room temperature and under agitation. Stripping ended with incubation with respective primary antibody, overnight at 4ºC with shaking. Serving as protein load control, membrane was re-probed with monoclonal anti-β-actin. Band density was measured with Image J – Fiji software, and then normalized with anti-β-actin load amount.

RNA analyses

RNA extraction

RNA extraction process is described above in “protein analyses”. To the Eppendorf containing RNA (top layer resulted from the first centrifugation), 500µl of 70% ethanol (v/v) was added. RNA was purified using a RNeasy Mini Kit (QIAGEN) and later quantified (Thermo Fisher Scientific NanoDrop™ 2000c). RNA was diluted to a final concentration of 1 µg/µl. RNA was stored at -80ºC.

Reverse transcription

1000ng of total RNA of each sample was converted into cDNA using iScript™ cDNA synthesis kit (BioRad Laboratories, Hercules, CA, USA) following manufacturer’s instructions.

Quantitative Real Time – Polymerase Chain Reaction

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was performed using CFX96TM real time machine (BioRad) with SsoAdvanced™ Universal SYBR® Green Supermix (BioRad). Reactions took place in 96 or 384 well microtiter plate. A master mix was prepared for each set of primers. Total reaction volume was 15µl, being 13,5µl of master mix and 1,5µl of template cDNA. Primers used in qRT-PCR process are listed on table 6. Aiming to achieve maximal cDNA amplification and with concordance with primer’s manufacturer, the cycling parameters were 1 cycle at 95ºC for 30sec, then 40 cycles at 95ºC for 5sec, annealing temperature, recommended by manufacturer, for 15sec. The process *terminus* was 1 cycle at 57ºC to 90ºC for 5sec (melting curve). GAPDH were use as “housekeeping” gene. Cycle threshold (Ct) values were automatically determined by CFXManager Software. Results of mRNA quantification are expressed relatively to “housekeeping” gene.

Table 6 - List of primers used in RT-qPCR analysis.

Primer	Forward Sequence	NCBI Reference Sequence	Manufacturer
POMC	CAGGACCTCACCACGGAAAG	NM_139326.2	Invitrogen
AgRP	TCCCAGAGTTCCCAGGTCTAA	NM_007427.3	Invitrogen
GHSR	CCGATAGAGTGACAGGCTTCTT	NM_177330.4	Invitrogen
LEP R	GGACTGAATTTCCAAAAGCCTGAA	NM_146146.2	Invitrogen
NPY Y1R	TGCTACTTCAAGATATACGTTCGC	NM_001113357.1	Invitrogen
NPY Y2R	TGGGTCCAGTTTTGTGCCAT	NM_023968.1	Invitrogen
NPY Y5R	GGGGCATCCCGAGGATTTTA	NM_012869.1	Invitrogen
TNF-α	CCCACGTCGTAGCAAACCA	NM_013693.3	Invitrogen

Results

Animals treated with ghrelin presented a higher weight loss, while the ingested food is similar in both treated and control groups

Known as "hunger hormone" or "feeding peptide", ghrelin is described to enhance appetite and to increase food intake (Wren, Seal et al. 2001). Thus, in this first part of the analyses we aimed to investigate if ghrelin treatment affected the weight of the animals, as well as the food intake. For that, animals from cohort 1 (*n=8*) were additionally divided in 2 subgroups: control (*n=4*) and experimental (*n=4*). Animals from cohort 2 were also divided in 2 similar groups: control (*n=9*) and experimental (*n=13*). Control groups were submitted to a daily subcutaneous injection of NaCl (0.9%), while experimental groups took a subcutaneous injection of ghrelin. We observed that treated animals presented a higher weight loss compared to control animals, although this difference was only statistically significant for cohort 1 (fig. 9) [cohort 1: control (0.43 ± 0.07, *n=3*); treated (-2.19g ± 0.83g, *n=4*) – P-value = 0.0453; cohort 2: control (-0.65g ± 0.43g, *n=9*); treated (-1.34g ± 0.32g, *n=13*) – P-value = NS (unpaired Student's t-test)]. Data were represented as mean ± SEM. [P-value < 0.05 (*)].

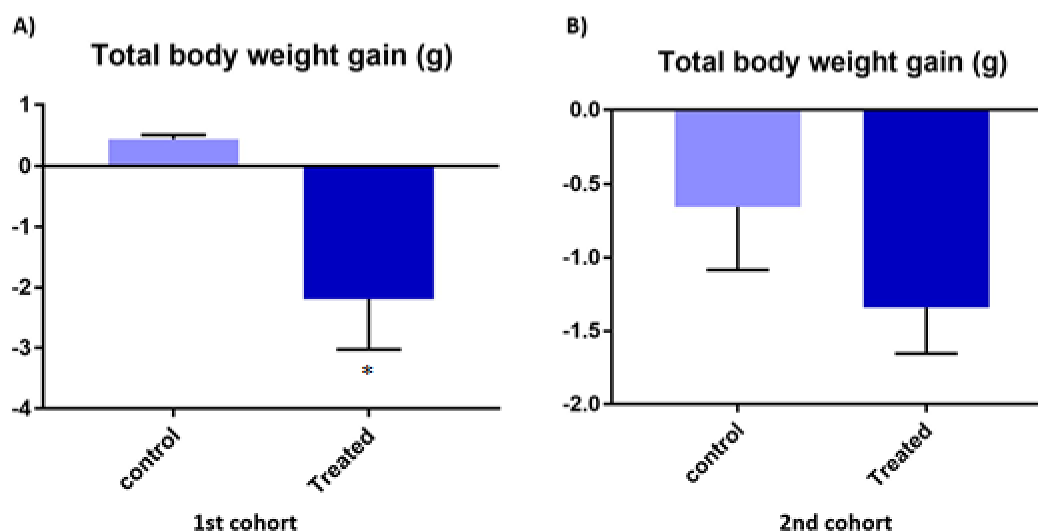


Figure 9 *- Total body weight gain throughout the experiment (g).* ***A)*** *short-term treatment [control (0.43g ± 0.07g, n=3); treated (-2.19g ± 0.83g, n=4) – P-value = 0.0453 (unpaired Student's t-test)];* ***B)*** *long-term treatment [control (-0.65g ± 0.43g, n=9); treated (-1.34g ± 0.32g, n=13) – P-value = NS (unpaired Student's t-test)].*

Despite being well established that ghrelin induces food intake (Callahan, Cummings et al. 2004), our results shown no significant differences of ingested food between control and treated animals (fig.10) [cohort 1: control (109.90g ± 11.87g, *n=4*); treated (110.70g ± 6.58g, *n=4*) – P-value = 0.0405 (unpaired Student's t-test); cohort 2: control (229.30 ± 9.22, *n=9*); treated (215.80g ± 5.57g, *n=13*) – P-value = NS (unpaired Student's t-test)]. Data were represented as mean ± SEM.

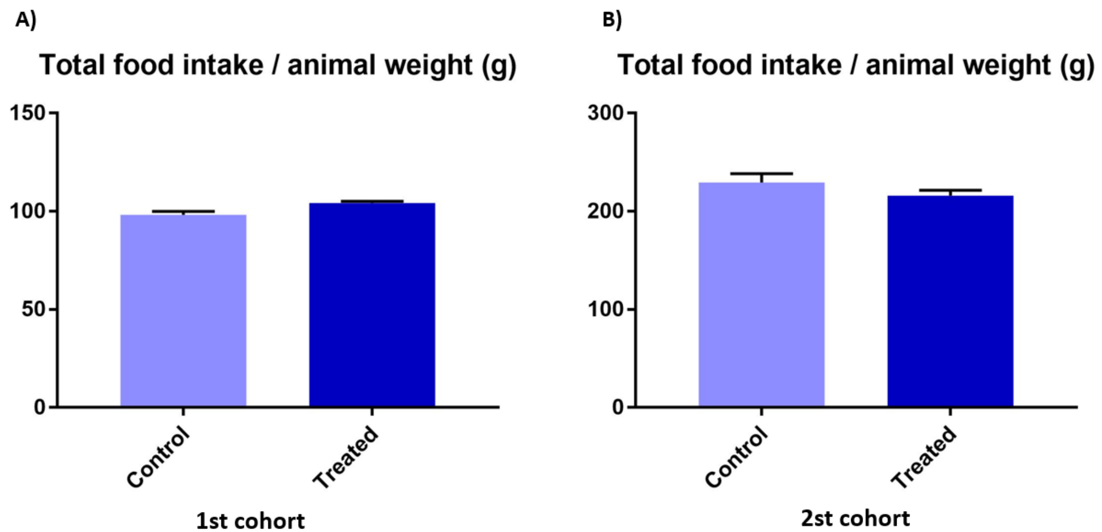


Figure 10 *- Total food intake per animal throughout the experiment (g).* ***A)*** *short-term treatment [control (109.90g ± 11.87g, n=4); treated (110.70g ± 6.58g, n=4) – P-value = 0.0405]* ***B)*** *long-term treatment [control (229.30g ± 9.22g, n=9); treated (215.80g ± 5.57g, n=13) – P-value = NS (unpaired Student's t-test)].*

Treated animals with ghrelin display similar liver weight, decreased spleen weight and increased WAT weight when compared to control animals

In the liver, ghrelin deletion prevents hepatic steatosis and, subsequently, decreases liver weight (Guillory, Jawanmardi et al. 2018). For that, we also analyzed the weight of different metabolic tissues and organs.

The results from the short-term treatment (cohort 1) show no significant difference between treated and control animals in the liver weight (fig. 11 A)) [control (50.73mg/g ± 1.21mg/g, *n= 4*); treated (53.81mg/g ± 2.66mg/g, *n=4*) – P-value = NS (unpaired Student's t-test)]. We also observed that treated animals have a propensity to have decreased spleen weight (fig. 11 B)) and increased WAT weight compared to control animals (fig. 11 C)) [spleen: control (3.32mg/g ± 0.39mg/g, *n=4*); treated (2.76mg/g ± 0.32mg/g, *n=3*) – P-value=NS; WAT: control (16.04mg/g ± 4.38mg/g, *n=4*); treated (27.83mg/g ± 7.07mg/g, *n=4*) – P-value=NS (unpaired Student's t-test)]. Data were represented as mean ± SEM.

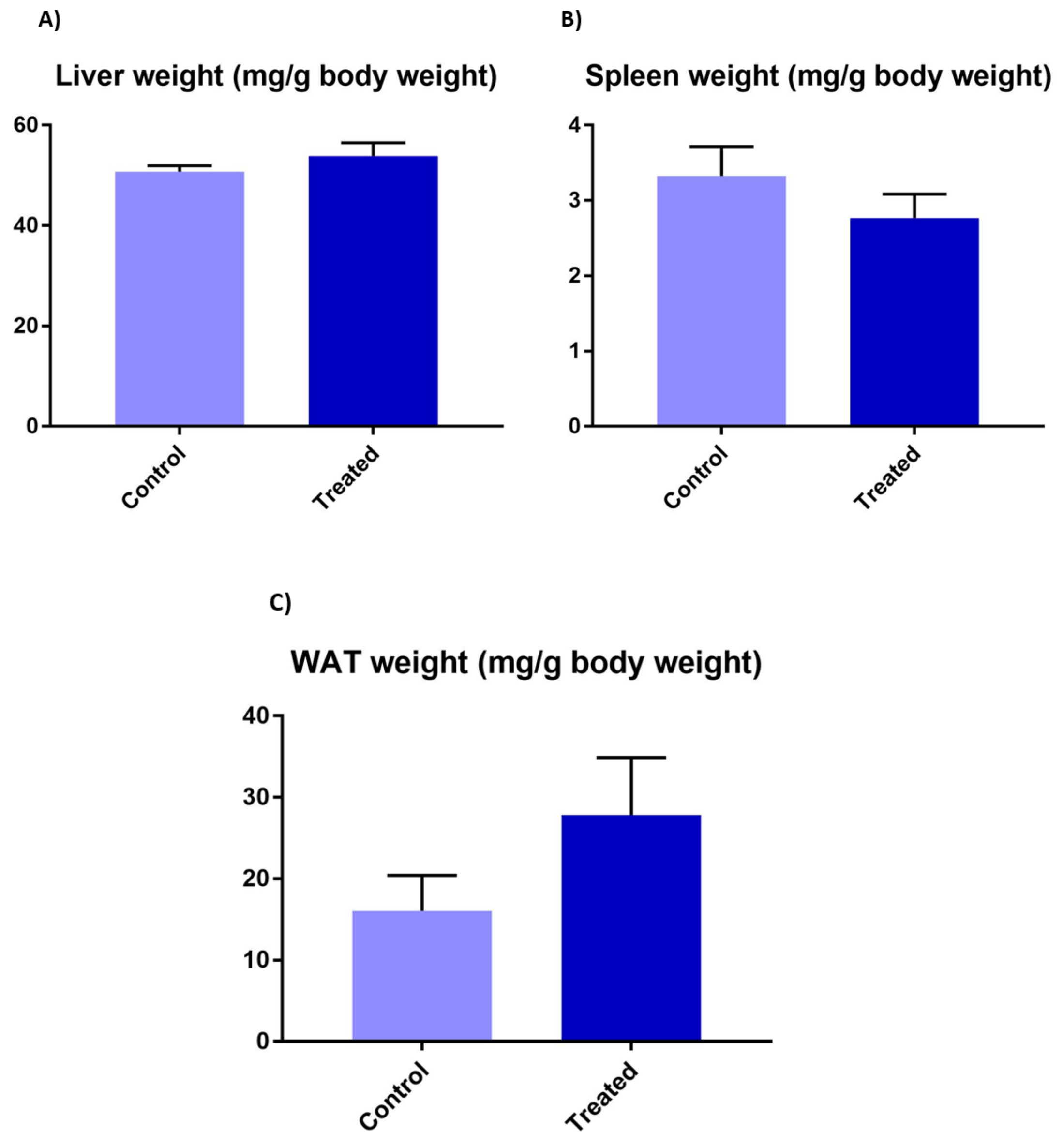


Figure 11 *- Short-term treated with ghrelin animals have similar liver weight, decreased spleen weight and increased WAT weight when compared to control animals.* ***A)*** *Liver weight (mg/g body weight) [control (50.73mg/g ± 1.21mg/g, n= 4); treated (53.81mg/g ± 2.66, n=4) – P-value = NS (unpaired Student's t-test)].* ***B)*** *Spleen weight (mg/g body weight) [control (3.32mg/g ± 0.39mg/g, n=4); treated (2.76mg/g ± 0.32mg/g, n=3) – P-value=NS (unpaired Student's t-test)].* ***C)*** *WAT weight (mg/g body weight) [control (16.04mg/g ± 4.38mg/g, n=4); treated (27.83mg/g ± 7.066mg/g, n=4) – P-value=NS (unpaired Student's t-test)].*

Behaviour

Treated animals show fewer rearing episodes and decreased rearing time compared with control animals

Rearing is considered an exploratory behaviour and is widely used to measure mice anxiety levels (Seibenhener and Wooten 2015). However, it is yet to be resolved if increased rearing is a signal of increased anxiety (Borta and Schwarting 2005) or if decreased rearing means higher anxiety levels (Costall, Jones et al. 1989).

Rearing behaviour was considered when mice stood in both hind paws in a vertical position.

When compared with control group, long-term treated animals showed fewer rearing episodes (fig. 12 A)) [control (52.11 ± 5.59 *n=9*); treated (38.58 ± 3.54, *n=12*) – P-value = 0.0456 (unpaired Student's t-test)] and decreased rearing time (fig. 12 B)) [control (53.62 ± 6.21 *n=9*); treated (37.28 ± 3.52, *n=12*) – P-value = 0.0250 (unpaired Student's t-test)], which might indicate a diminished exploratory behaviour and an enhanced anxiety level. Data were represented as mean ± SEM. [P-value < 0.05 (*)].

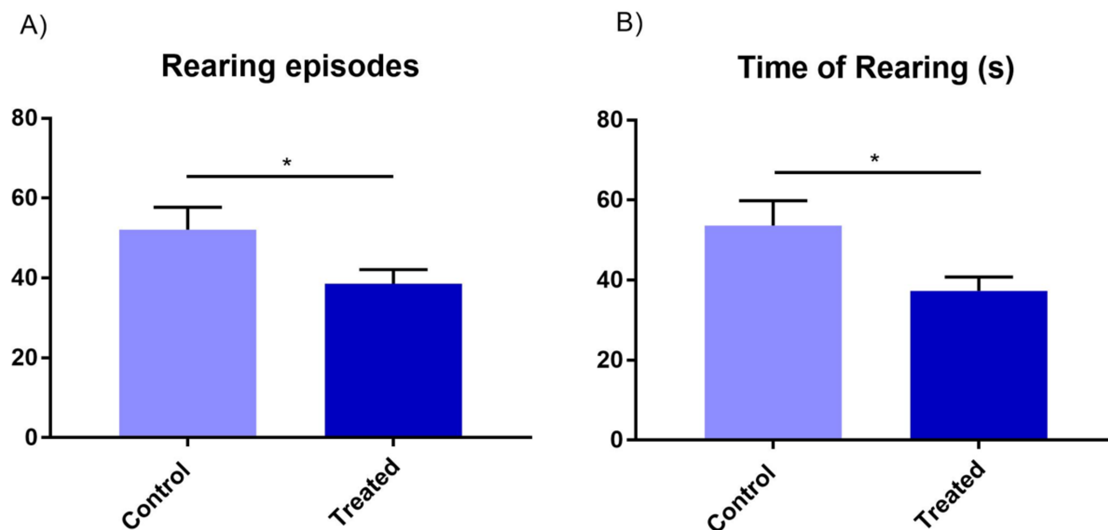


***Figure** 12 - Long-term treated animals with ghrelin show fewer rearing episodes and decreased rearing time compared with control animals. **A)** Rearing episodes [control (52.11 ± 5.59 n=9); treated (38.58 ± 3.54, n=12) – P-value = 0.0456 (unpaired Student's t-test)]. **B)** Time of Rearing (s) control (53.62 ± 6.21 n=9); treated (37.28 ± 3.52, n=12) – P-value = 0.0250 (unpaired Student's t-test)].*

Treated animals shown fewer grooming episodes and decreased grooming time compared with control animals

Grooming is an innate behaviour, very common in rodents, involved in hygiene maintenance, thermoregulation and social communication (Spruijt, van Hooff et al. 1992); (Kalueff, Stewart et al. 2016). Self-grooming tends to increase in stressful situations (Kalueff, Stewart et al. 2016), making grooming behaviour a useful tool to study mice stress and anxiety.

When compared with control group, long-term treated animals with ghrelin tends to have fewer grooming episodes (fig.13 A)) [control (9.78 ± 2.21 *n=9*); treated (6.33 ± 0.62, *n=12*) – P-value = 0.1059 (unpaired Student's t-test)] and reduced time spend grooming (fig. 13 B)) [control (20.44 ± 1.05 *n=8*); treated (16.57 ± 1.54, *n=12*) – P-value =0.0797 (unpaired Student's

t-test)]. Data were represented as mean ± SEM. This data suggests that treated mice have decreased stress levels when compared with control animal.

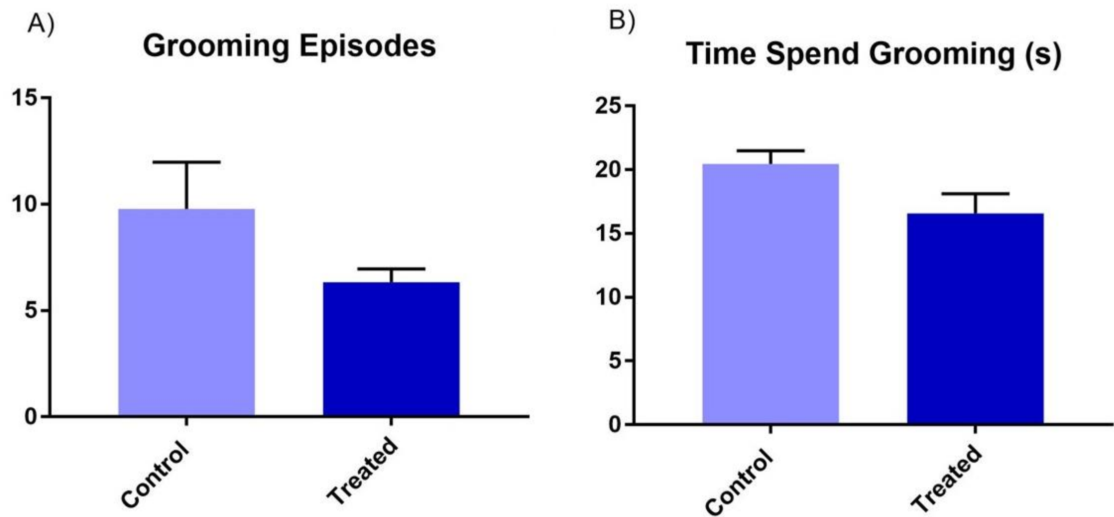


Figure 13 *– Long-term treated animals with ghrelin show fewer grooming episodes and decreased grooming time compared with control animals.* ***A)*** *Grooming Episodes [control (9.78 ± 2.21 n=9); treated (6.33 ± 0.62, n=12) – P-value = 0.1059 (unpaired Student's t-test)].* ***B)*** *Time Spend Grooming (s) [control (20.44 ± 1.05 n=8); treated (16.57 ± 1.54, n=12) – P-value =0.0797 (unpaired Student's t-test)].*

Other parameters evaluated in the open field experiment like total distance, mean speed, immobile episodes and time spend immobile, number of line crossing, entries in the middle and time spend in the middle showed no significant differences between control group and treated group are summarized in the following table (table 7; annex 2, 3, 4 and 5).

Table 7 - Overall results of parameters analysed in open field experiment: Total distance, mean speed, number of immobile episodes, time spend immobile, number of crossing lines, number of entries in the middle, time spend in the middle, number of grooming's, time spend grooming, number of rearing and time spend rearing.

	Control Group	**Treated Group**	**p-value**
Total Distance (m)	30.57 ± 3.48 (*n=9*)	27.73 ± 2.58 (*n=12*)	0.5112
Mean Speed (m/s)	0.051 ± 0.006 (*n=9*)	0.046 ± 0.004 (*n=12*)	0.05123
Number of immobile episodes	36.2 ± 2.4 (*n=9*)	36.5 ± 2.3 (*n=12*)	0.9357
Time spend immobile (s)	201.2 ± 21.6 (*n=9*)	225.5 ± 23.9 (*n=12*)	0.4761
Number of crossing lines	223.9 ± 23.1 (*n=9*)	202.5 ± 17.7 (*n=12*)	0.4629
Number of entries in the middle	20.67 ± 3.60 (*n=9*)	19.58 ± 1.8 (*n=12*)	0.7749
Time spend in the middle (s)	32.72 ± 7.38 (*n=9*)	28.98 ± 5.52 (*n=12*)	0.6824
Number of grooming's	9.78 ± 2.21 (*n=9*)	6.33 ± 0.62 (*n=12*)	0.1059
Time spend grooming (s)	20.44 ± 1.05 (*n=8*)	16.57 ± 1.54 (*n=12*)	0.0797
Number of rearing's	52.11 ± 5.59 (*n=9*)	38.58 ± 3.54 (*n=12*)	0.0456 (*)
Time spend rearing (s)	53.62 ± 6.21 (*n=9*)	37.28 ± 3.52 (*n=12*)	0.0250 (*)

Treated mice with ghrelin have less signs of adipocytes death in BAT

The main function of BAT is to use lipids to generate heat (Cannon and Nedergaard 2004). During a cold stimulus, sympathetic nervous system (SNS) induces the release of norepinephrine into BAT, which activates β3-adrenergic receptor (β3-AR), uncoupling protein 1 (UCP1) is mobilized to mitochondria, lipolysis occurs and, subsequently, thermogenesis (Kozak, Koza et al. 2010). Therefore, we analyzed the integrity of BAT through histology. We observed that, in short-term treatment, treated animals have fewer dead adipocytes (fig 14, A) in BAT than control animals (fig. 14 B), suggesting a more functional BAT.

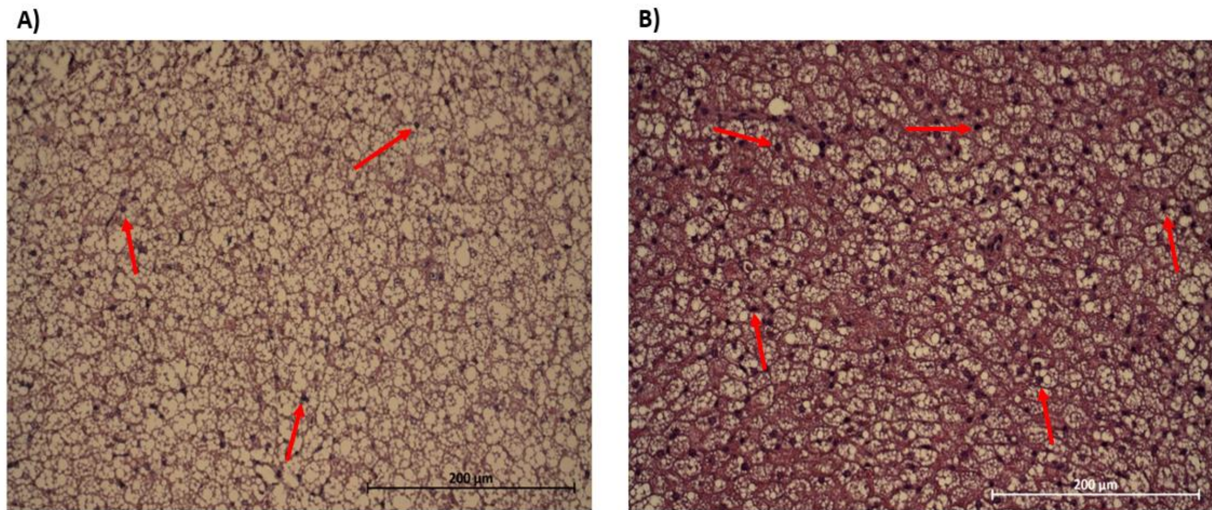


Figure 14 - Treated mice with ghrelin have fewer dead adipocytes in BAT. Dead adipocytes are represented as black dots and some are highlighted with red arrow. ***A)*** *Treated;* ***B)*** *Control.*

Treated mice with ghrelin have a larger adipocyte in WAT

WAT main function is to accumulate lipids (Kuryszko, Slawuta et al. 2016), being also important in glucose and lipid metabolism (Cancello, Tounian et al. 2004). Thus, we analyzed the area of WAT adipocytes in both groups.

When compared with control group, short-term treated animals with ghrelin showed larger adipocyte area (fig. 15) [control (5187µm² ± 558 µm² *n=4*); treated (7637 µm² ± 410 µm², *n=3*) – P-value = 0.0217 (unpaired Student's t-test)]. Data were represented as mean ± SEM. [P-value < 0.05 (*)].

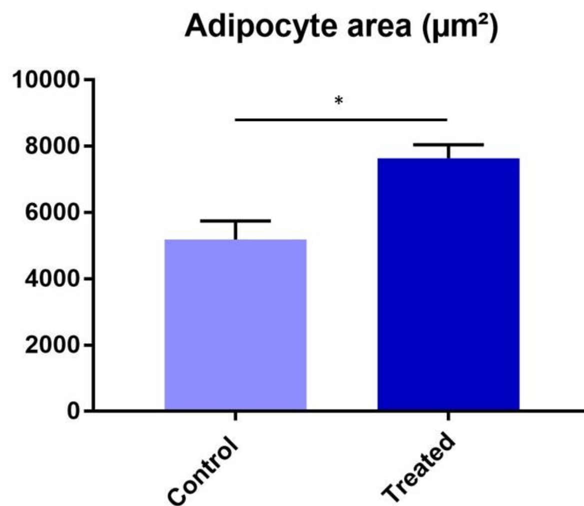


***Figure** 15 - Treated animals with ghrelin have a larger adipocyte area (µm²) in WAT in short-term treatment [control (5187 µm² ± 558 µm² n=4); treated (7637 µm² ± 410 µm², n=3) – P-value = 0.0217 (unpaired Student's t-test)].*

Regarding signs of inflammation in WAT, we do not observe significative differences between treated and control animals (annex 6).

Treated animals with ghrelin have a tendency of having larger pancreatic area and less dead cells

Pancreas main function is to regulate whole-body blood glucose level. This regulation occurs due to segregation of pancreatic hormones like insulin and glucagon from islets of Langerhans (Alamri, Shin et al. 2016). Both ghrelin and GHSR are expressed in islets of Langerhans, thus suggesting an important role of ghrelin in insulin and glucagon segregation (Muller, Nogueiras et al. 2015).

Although not significant, we observed that short-term treated animals with ghrelin present fewer dead cells in islets of Langerhans than control animals (fig.16 B) and fig. 17) [control (130.7µm² ± 66.1µm², *n=3*); treated (65.0µm² ± 17.9 µm², *n=4*) – P-value = 0.3180 (unpaired Student's t-test)].

We also observed that short-term treated animals have a tendency to present larger pancreatic islets area (fig. 16 C) and fig. 17) [control (121966 ± 76670 *n=3*); treated (192290 ± 63104, *n=4*) – P-value = 0.5069 (unpaired Student's t-test)]. Data were represented as mean ± SEM.

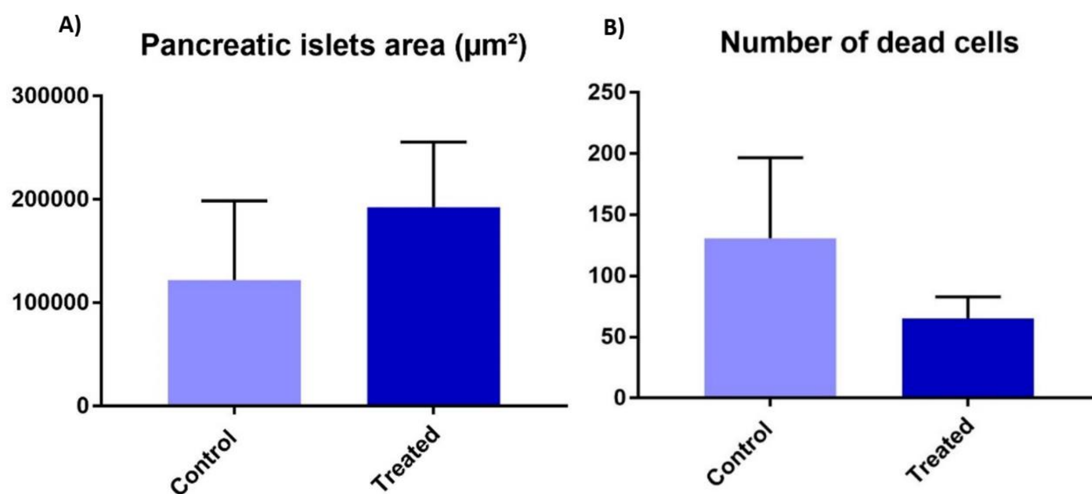


***Figure** 16 - Treated animals have larger pancreatic area and less dead cells **A)** Pancreatic islets area (µm²) [control (12196µm² ± 76670µm² n=3); treated (192290µm² ± 63104µm², n=4) – P-value = 0.5069 (unpaired Student's t-test)] **B)** Number of dead adipocytes pancreatic islets of Langerhans [control (130.7 ± 66.1 n=3); treated (65.0 ± 17.9, n=4) – P-value = 0.3180 (unpaired Student's t-test)].*

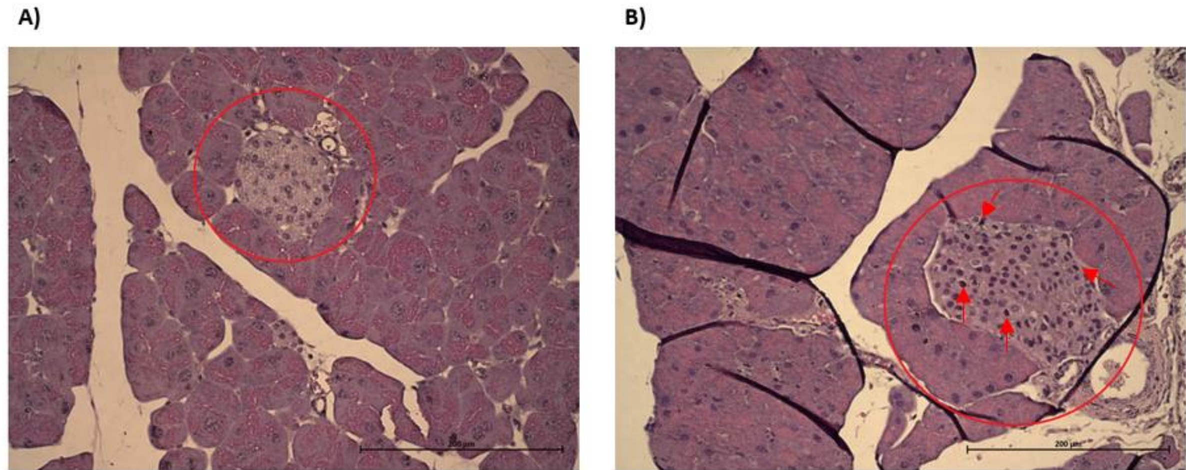


Figure 17 - Representative images of dead cells in pancreatic islets of Langerhans in short-term treatment with ghrelin. ***A)*** *Treated;* ***B)*** *Control.*

Both treated and control animals have similar liver histology

Guillory and colleagues (Guillory, Jawanmardi et al. 2018) reported that ghrelin deletion prevents liver age-related steatosis. Controversially, others (Barazzoni, Semolic et al. 2014) reported that ghrelin administration lowers inflammation and reduces triglycerides accumulation in rats. Moreover, ghrelin can have a protective effect on liver fibrosis (Mao, Zhang et al. 2015).

Despite all these pieces of evidence we do not observe any differences between control and treated animals concerning the liver histology (annex 7).

qRT-PCR analyses

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) is a molecular biology technique that allows monitoring cDNA amplification of a target DNA sequence (normally a gene) (Bustin, Benes et al. 2009). With this technique, we aimed to evaluate how ghrelin treatments affects gene expression in the hypothalamus.

We observed that, in the short-term treatment with ghrelin, treated animals have a tendency to present higher expression of orexigenic-related genes (NPY Y1R, NPY Y2R, NPY Y5R, AgRP) (fig. 18) and lower expression of anorexigenic genes (POMC) (fig. 19) when compared to control animals [fig. 18 A): control (100.0 ± 18.5 *n=4*); treated (141.3 ± 26.3, *n=4*) – P-value = 0.2458 (unpaired Student's t-test); B): control (100.0 ± 21.0 *n=4*); treated (151.6. ± 55.2, *n=4*) – P-value = 0.4160 (unpaired Student's t-test); C): control (100.42.4 ± *n=4*); treated (87.3 ± 10.8, *n=4*) – P-value = 0.7811 (unpaired Student's t-test); D): control (100.0 ± 27.26 *n=4*); treated (167.2 ± 37.5, *n=4*) – P-value = 0.1975 (unpaired Student's t-test)] [fig. 19: control (100.0 ± 67.5 *n=4*); treated (35.0 ± 3.1, *n=3*) – P-value = 0.4530 (unpaired Student's t-test)]. Data were represented as mean ± SEM.

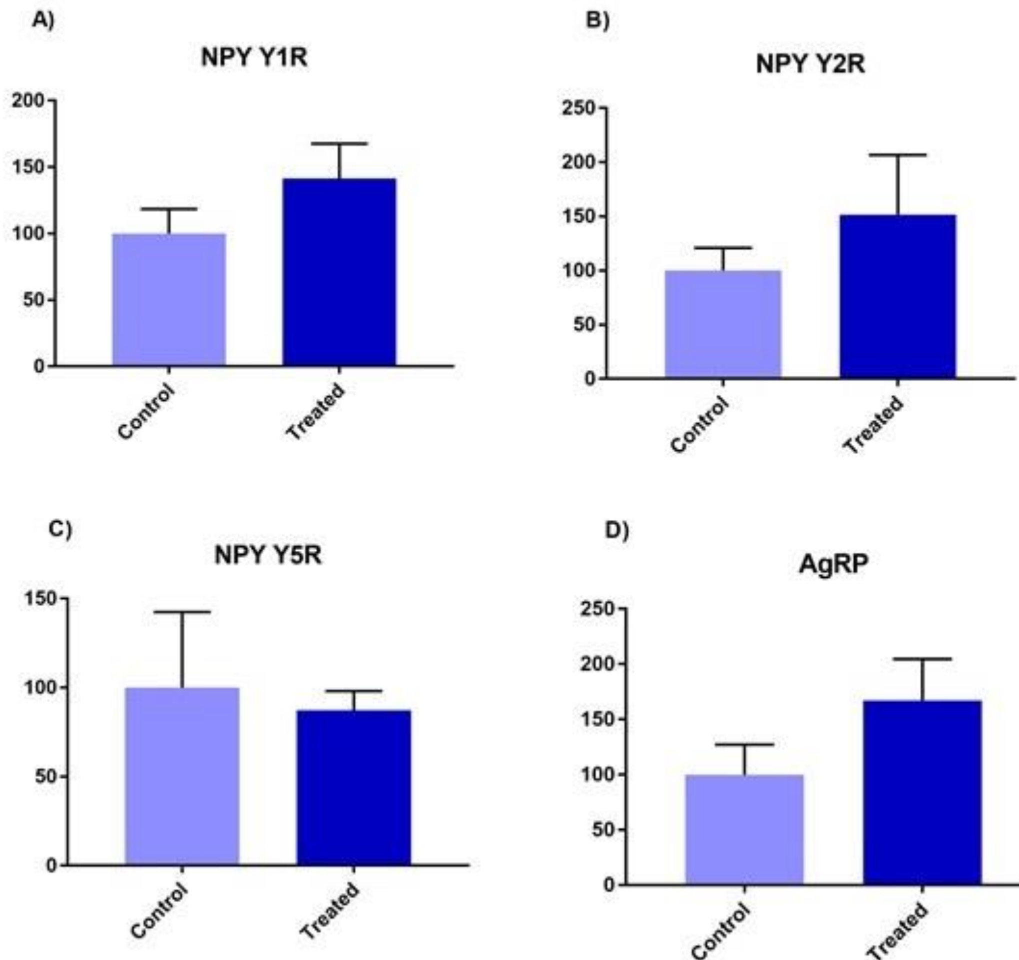


***Figure** 18 - mRNA expression of orexigenic-related neuropeptides in the hypothalamus. **A)** NPY Y1R [control (100.0 ± 18.5 n=4); treated (141.3 ± 26.3, n=4) – P-value = 0.2458 (unpaired Student's t-test)]. **B)** NPY Y2R [control (100.0 ± 21.0 n=4); treated (151.6. ± 55.2, n=4) – P-value = 0.4160 (unpaired Student's t-test)]. **C)** NPY Y5R [control (100 ± 42.4 n=4); treated (87.3 ± 10.8, n=4) – P-value = 0.7811 (unpaired Student's t-test)]. **D)** AgRP [control (100.0 ± 27.26 n=4); treated (167.2 ± 37.5, n=4) – P-value = 0.1975 (unpaired Student's t-test)].*

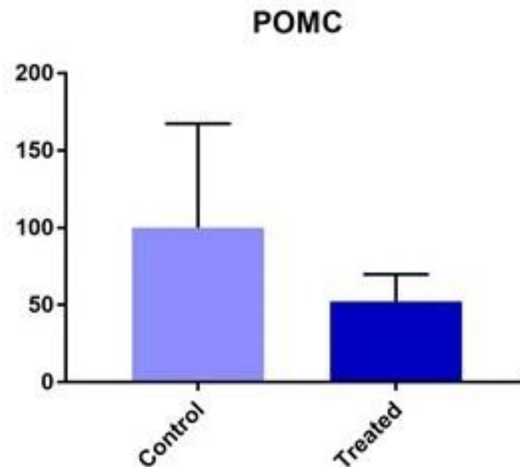


Figure 19 - mRNA expression of anorexigenic neuropeptide POMC in the hypothalamus [(control (100.0 ± 67.5 n=4); treated (35.0 ± 3.1, n=3) – P-value = 0.4530 (unpaired Student's t-test)].

We also measured mRNA expression in the hypothalamus of GHSR and Lep R genes and we observed that GHSR expression is similar in both treated and control animals while Lep R is significantly more expressed in treated animals (fig. 20). [Fig. 20 A): control (100.0 ± 17.3 *n=4*); treated (93.3 ± 22.8, *n=4*) – P-value = 0.8218 (unpaired Student's t-test); B): control (100.0 ± 34.7 *n=4*); treated (250.3 ± 38.0, *n=4*) – P-value = 0.0266 (unpaired Student's t-test)]. Data were represented as mean ± SEM. [P-value < 0.05 (*)].

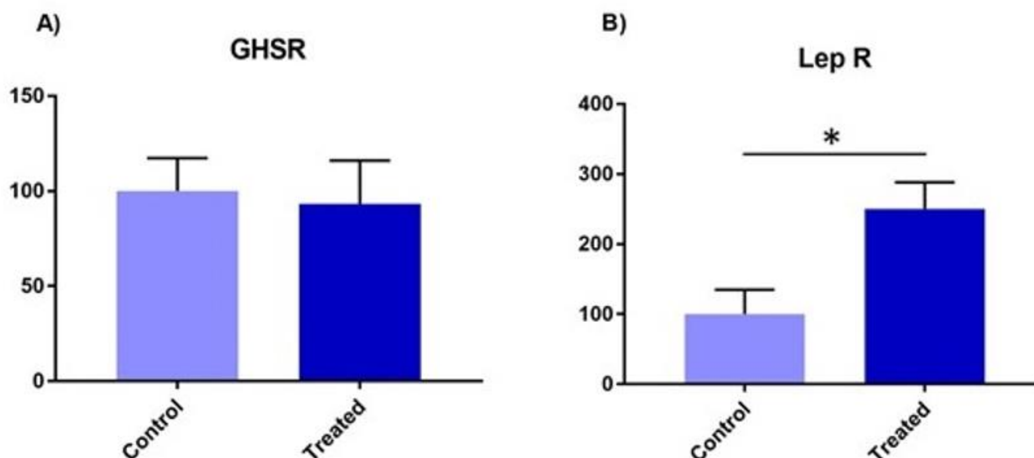


Figure 20 - mRNA expression of metabolic mediators in hypothalamus. ***A)*** *GHSR [control (100.0 ± 17.3 n=4); treated (93.3 ± 22.8, n=4) – P-value = 0.8218 (unpaired Student's t-test)].* ***B)*** *Lep R [control (100.0 ± 34.7 n=4); treated (250.3 ± 38.0, n=4) – P-value = 0.0266 (unpaired Student's t-test)].*

In order to analyse hypothalamus inflammation, we measured TNF-α mRNA expression. TNF-α is an inflammatory cytokine that is produced in acute inflammation process leading to necrosis and apoptosis (Idriss and Naismith 2000). We observed that, in short-term treatment,

treated animals have significantly less TNF-α mRNA expression when compared to control animals (fig. 21) [control (100.0 ± 18.7 *n=4*); treated (21.93 ± 3.142, *n=3*) – P-value = 0.0171 (unpaired Student's t-test)]. Data were represented as mean ± SEM. [P-value < 0.05 (*)].

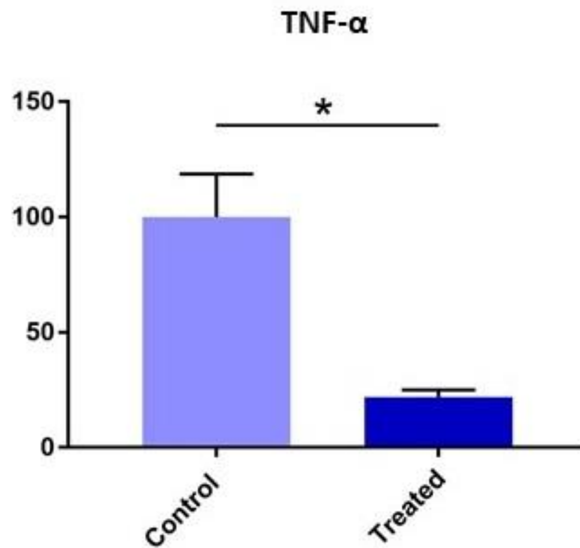
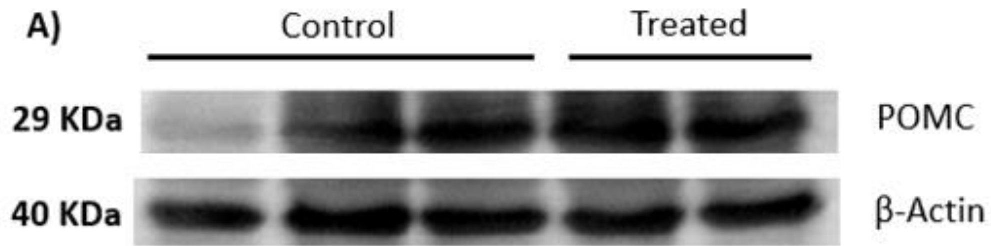


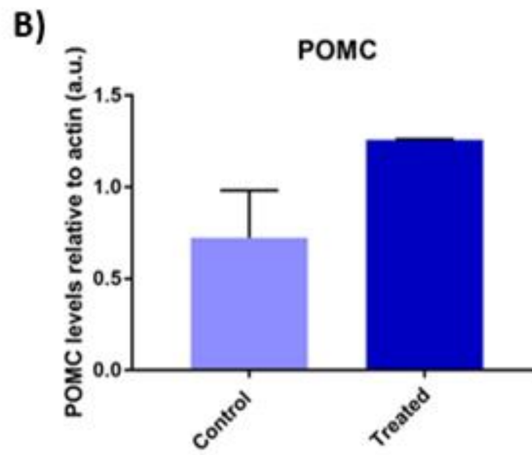
***Figure** 21 - mRNA expression of TNF-α in hypothalamus [control (100.0 ± 18.7 n=4); treated (21.93 ± 3.142, n=3) – P-value = 0.0171 (unpaired Student's t-test)].*

Treated animals with ghrelin have higher levels of POMC, AgRP and IL6 proteins in the hypothalamus

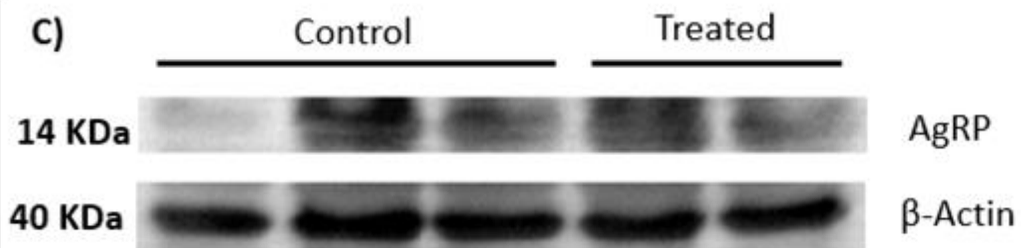
In order to evaluate the presence of POMC, AgRP and IL6 proteins in mice's hypothalamus, we performed WB analyses. We used β-Actin as loading control. Optical density of the target proteins bands was normalized with optical density of β-Actin band. Short-term treated mice with ghrelin display a higher level of POMC (fig. 22 A) and B)) proteins in hypothalamus and similar levels of AgRP (fig. 22 C) and D)) and IL6 (fig. 22 E) and F)) when compared to control animals [fig. 22: B): control (0.723 ± 0.259, *n=3*); treated (1.259 ± 0.003, *n=2*) – P-value = 0.2070 (unpaired Student's t-test); D): control (0.4945 ± 0.165, *n=3*); treated (0.6228 ± 0.144, *n=2*) – P-value = 0.6282 (unpaired Student's t-test); F): control (0.9985 ± 0.1383 *n=3*); treated (1.211 ± 0.018, *n=2*) – P-value = 0.3202 (unpaired Student's t-test)]. Data were represented as mean ± SEM.

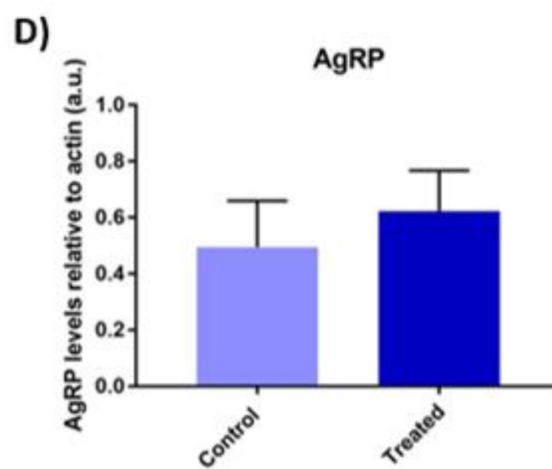
POMC





AgRP





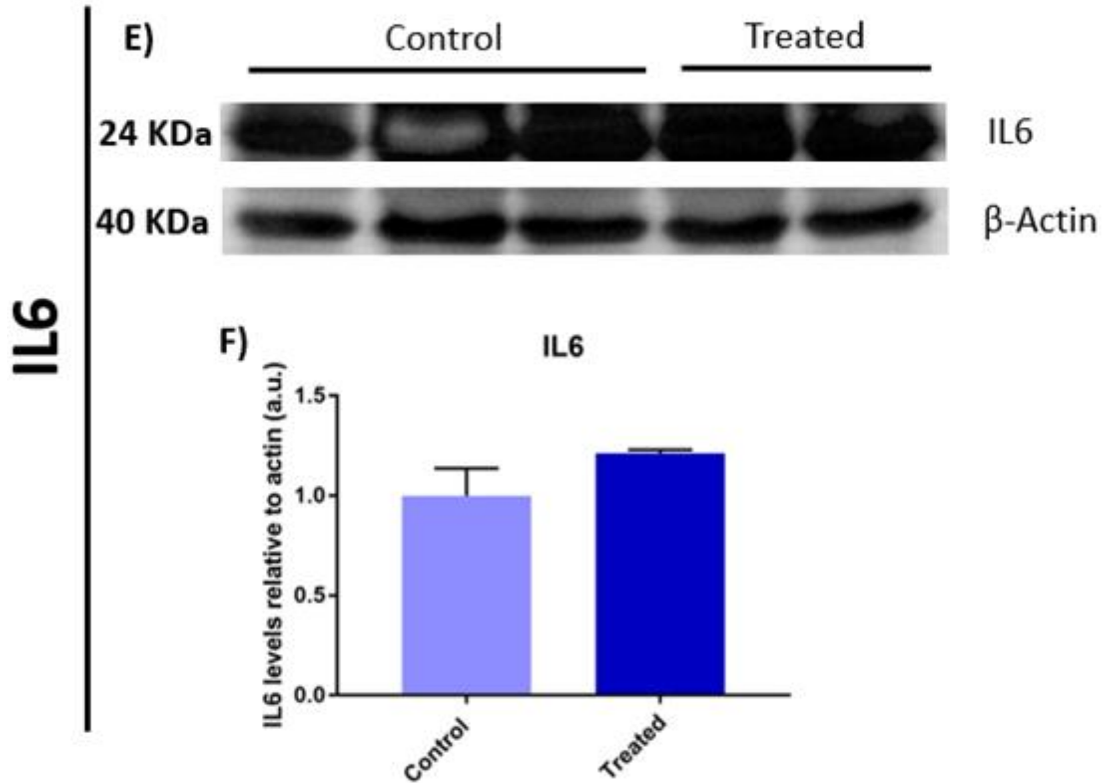


***Figure** 22* - Western-Blot analyses. Treated animals with ghrelin have higher levels of POMC, AgRP and IL6 proteins in the hypothalamus. **A)** POMC gel band at 29KDa in both treated and control animals. **B)** POMC levels relative to β-Actin (a.u.) [control (0.723 ± 0.259, n=3); treated (1.259 ± 0.003, n=2) – P-value = 0.2070 (unpaired Student's t-test)]. Data were measured as optical density in Image J – Fiji software. **C)** AgRP gel band at 14KDa in both treated and control animals. **D)** AgRP levels relative to β-Actin (a.u.) [control (0.4945 ± 0.165, n=3); treated (0.6228 ± 0.144, n=2) – P-value = 0.6282 (unpaired Student's t-test)]. Data were measured as optical density in Image J – Fiji software. **E)** IL6 gel band at 24KDa in both treated and control animals. **F)** IL6 levels relative to β-Actin (a.u.) [control (0.9985 ± 0.1383 n=3); treated (1.211 ± 0.018, n=2) – P-value = 0.3202 (unpaired Student's t-test)]. Data were measured as optical density in Image J – Fiji software.

Discussion

Ghrelin treatment induces body weight loss

Ghrelin is a well-studied hormone that induce food intake and, that could lead subsequently, to an increase in body weight (Callahan, Cummings et al. 2004). However, literature mainly focus on young and middle age mice, and studies of ghrelin's role in advanced aged mice are lacking. In fact, we observed that in both cohorts (short-term and long-term treatments), treated animals with ghrelin presented a higher weight loss compared to control animals (fig.9). This difference is particularly significant in the short-term treatment. In fact, control animals presented a weight gain while treated animals had a weight loss. Although not significant, this tendency was also observed in the long-term treatment. Because ghrelin induces food intake, it is expectable that weight variation is correlated with food intake. However, we observed no differences in food intake between control and treated groups, in both short-term and long-term cohorts (fig.10). This data suggests that ghrelin can act on body weight regulation without affecting feeding behaviour. (Shiiya, Nakazato et al. 2002) came to a similar conclusion when they observed that individuals with high levels of circulating ghrelin were not obese and individuals with low levels of circulating ghrelin were not anorectic. However, the mechanisms underlying this correlation requires further investigation.

Additionally, we observed that organs weights were similar in both control and treated animals, suggesting that weight loss does not come from any specific organ (fig. 11 A) and B)). In fact, treated animals had a higher WAT weight when compared with control group (Fig. 11 C)).

In sum, ghrelin induces increased body weight in young and adult mice but tends to decrease body weight in old mice. This tendency is not related to food intake.

Ghrelin treatment induces less stress and anxiety in mice in open field test

It is well established that mice behavior analyses can provide fundamental clues about stress and anxiety levels (Seibenhener and Wooten 2015). Unfortunately, scientific community cannot agree if certain specific behaviors like rearing and grooming means increased or decreased stress/anxiety levels. Our knowledge and expertise in analyzing mice behavior lead us to believe that exacerbated rearing and grooming is signal of stress and anxiety. These thoughts are in line with (Sturman, Germain et al. 2018), as they observed that rearing is sensitive to acute stress and anxiety. Plus, (Nosek, Dennis et al. 2008) and (Spruijt, van Hooff et al. 1992) have related grooming activity with stress and anxiety state.

In the open field test, we observed that in the short-term treated animals with ghrelin have a significant decrease in the rearing behavior comparing to control animals (both rearing episodes and time spend rearing) (fig. 12 A) and B)). Knowing that rearing is an exploratory behavior (Seibenhener and Wooten 2015), our data indicates that treated animals feel less need to explore the environment beyond the arena. In addition, treated mice with ghrelin also shown less grooming behavior (fig. 13). Together, these results suggest that treated mice feel more comfortable inside the arena, and therefore, less stressed and anxious. Nevertheless, this data is unable to explain why treated have a higher weight loss, since feeding behavior is similar in both groups.

BAT histology

According to (Lin, Lee et al. 2014), thermogenic impairment occurs with aging. However, this impairment might be mitigated by suppressing ghrelin signaling (via GHSR). Controversially, we observed that a short-term ghrelin treatment seems to reduce the number of dead adipocytes (fig. 14), thus preventing BAT dysfunction. Unfortunately, our data is insufficient to evaluate BAT function. In order to evaluate BAT function, we could evaluate the expression of pro-inflammatory genes like TNF-α and IL-6 and the BAT-specific protein UCP-1. In addition, evaluate by Western-Blot the presence of ghrelin in BAT and correlate with the expression of pro-inflammatory genes. We hypothesized that ghrelin can is able to induce thermogenic impairment, however, further investigation is required. On the other hand, ghrelin is able to prevent adipocyte death. Therefore, the exact mechanism of ghrelin action requires further investigation, although we hypothesize that ghrelin acts in BAT throughout peripheric mediators like UCP-1.

WAT histology

As represented in fig. 15, WAT adipocyte area is significantly larger in treated animals with ghrelin when compared with control animals. This area differences might explain the fact that treated animals also have a heavier WAT than control animals (fig. 11 C)). (Davies, Kotokorpi et al. 2009) also came to similar conclusion, thus supporting our data. They suggest that adipocyte accumulation and enlargement is because ghrelin inhibits lipolysis and reduce lipids exportation. Plus, WAT-specific mediator of lipogenesis, LXRα (Schultz, Tu et al. 2000) and fatty acid synthase, remained unaffected. Alternatively, ghrelin enhances substrate uptake. For the authors, the most reasonable option is that ghrelin diminishes lipid exportation because reduces to half the expression of ABCG1, the principal mediator of cholesterol efflux (Vaughan and Oram 2005). According to (Pereira, da Silva et al. 2017), adipocyte disruption means WAT inflammation. However, we do not observe inflammation improvement of treated animals when compared to control animals (annex 6).

Pancreas histology

As presented in results, we found that treated animals with ghrelin have larger pancreatic islets area (fig. 16 A)) and less dead cells in pancreatic islets (fig. 16 B)) when compared to control group. Because pancreatic islets are so important to maintain glucose homeostasis, our results suggest that ghrelin short-term treatment seems to improve pancreatic functions. Unfortunately, our study lacks GTT and ITT tests to supports our hypotheses.

Liver histology

Several reports reveal a correlation between ghrelin and liver function. For example, (Guillory, Jawanmardi et al. 2018) reported that ghrelin deletion prevents hepatic steatosis and, consequently, reduces liver weight. In this line of thoughts, ghrelin administration should increase hepatic steatosis and liver weight. Our results show no differences in hepatic steatosis between treated and control group (annex 7). In addition, we observed no differences in liver weights (fig. 11 A)). (Barazzoni, Semolic et al. 2014) stated that ghrelin lowers liver

inflammation. Unfortunately, we lack inflammation biomarkers data in the liver, like TNF-α, to evaluate liver inflammation.

Molecular analyses

Because ghrelin plays a major role in food intake (Callahan, Cummings et al. 2004), it is expected that, somehow, ghrelin might influence expression of genes related to food intake. As mentioned in the results section, we observed that ghrelin short-term treatment was able to raise mRNA expression of orexigenic genes in the hypothalamus, namely NPY receptors Y1 and Y2 and AgRP (fig. 18). Although not statistical significant (P-value = 0.1975), the fact that ghrelin short-term treated animals have a higher expression of AgRP in hypothalamus is very relevant since AgRP is strongly regulated by ghrelin (Bewick, Gardiner et al. 2005). This association is well described in literature but for us, this means that our approach seems to be able to induce an effect in the hypothalamus. Another important player in food intake regulation is leptin. While ghrelin positively regulates NPY/AgRP expression, leptin negatively regulates NPY/AgRP expression (Elmquist, Coppari et al. 2005). According to literature (Elmquist, Coppari et al. 2005), leptin regulates NPY/AgRP and not the other way around. However, we observed that ghrelin treated animals have lower expression of leptin receptors (fig. 20 B). The specific correlation between increased ghrelin levels and decreased leptin receptors remains to be resolved. We also observed that anorexigenic gene POMC have a lower expression in the hypothalamus in ghrelin treated animals (fig. 19). Our results are supported by (Minor, Chang et al. 2009) report, where ghrelin induces NPY/AgRP expression and inhibits POMC/CART (fig. 3). In GHSR mRNA expression, we do not observe any differences between control and treated animals (fig. 20 A)). Although ghrelin specifically binds to GHSR, this data suggests that GHSR expression in hypothalamus is not directly related to circulatory levels of ghrelin in blood plasma.

Being an inflammatory cytokine, TNF-α is an important biomarker to access tissue inflammation. We observed that ghrelin treated animals have less TNF-α mRNA expression when compared to control animals (fig. 21 A)). This data suggests that administration of ghrelin significantly decreases hypothalamus inflammation. The mechanism underlying this correlation remains unclear and requires further investigation.

In the hypothalamus, we observed that POMC protein levels are higher in treated mice (fig. 22 A) and B)). This is an interesting result because we noted that mRNA expression levels are lower in treated mice. mRNA expression is not necessarily proportional to protein concentration. If a protein has a lower half-life time, it is required a higher rate of mRNA expression to maintain protein level at a certain concentration or vice-versa. This feedback mechanism can explain why treated animals have a higher POMC protein concentration but a lower POMC mRNA expression levels.

Although not significant, treated animals have slightly higher levels of AgRP in hypothalamus (fig. 22 C) and D)). This result comes in line with qRT-PCR results, where we observed higher levels of AgRP mRNA expression (fig. 18 D)). These results were expected because AgRP is strongly regulated by ghrelin in a positive manner.

IL6 is an interleukin that acts as pro-inflammatory cytokine and anti-inflammatory myokine (Ferguson-Smith, Chen et al. 1988). As a pro-inflammatory cytokine, IL6 is able to cross BBB, reach the hypothalamus and mediate the acute phase of inflammation (Banks, Kastin et al. 1994).

In general, our results are consistent with literature where ghrelin induces expression of orexigenic genes and inhibits expression of anorexigenic genes. However, and to understand the underlying mechanisms, it is required to perform molecular analysis in organs other than hypothalamus, namely in WAT, BAT and pancreas.

Conclusion

The administration of ghrelin can, in fact, have an effect in the whole-body metabolism and thus improve the hallmarks of aging. The impact of ghrelin in whole-body of our study is summarized in table 8.

Our histology analysis showed that ghrelin treatment reduces cell death both in BAT and Pancreas, thus improving function. We believe that this function improvement is related with mitochondrial improve of function, but more investigation is required. Loss of proteostasis is another primary hallmark of aging. Recent reports have showed that hypothalamus inflammation is widely related with loss of proteostasis. We observed that hypothalamus inflammation is significatively reduced in treated animals. We hypothesize that ghrelin is able to, somehow, restore proteostasis.

Nutrient sensing is an antagonistic hallmark; thus, it is hard to achieve a correct evaluation of its effect because an enhanced nutrient sensing can lead to exacerbated cell growth, thus augmenting systemic damage. In the other hand, a faulty nutrient sensing system can lead to extended lifespan as metabolism and cell growth are slower, therefore leading to decreased systemic cell damage. Our data showed that treated animals have an improved pancreatic function. Enhanced pancreatic function is important to achieve a proper regulation of insulin, thus a correct nutrient sensing.

Table 8 *- Role of ghrelin in the hallmarks of aging.*

	Hallmarks of aging	**Ghrelin effect**
Primary Hallmarks	Mitochondrial dysfunction	BAT: Less signs of adipocyte death Pancreas: Less dead cells
	Defective proteostasis	Significantly reduced inflammation in hypothalamus
Antagonistic Hallmarks	Nutrient sensing	Improved pancreas function

Ghrelin treatment can, in fact, play a role in the whole-body metabolism and homeostasis. In addition, ghrelin can improve aging hallmarks such as mitochondrial dysfunction, proteostasis and nutrient sensing, making ghrelin a possible therapeutical approach in order to extend lifespan.

Future perspectives

To complete this study, we aim to evaluate mice survival curve while submitted to ghrelin treatment. This test would allow us to understand if all the improvements we saw in this study can actually have measurable effects in mice lifespan.

Other important point to future evaluation is how ghrelin is able to induce weight loss without affecting feeding behaviour. A possible approach to do this evaluation is identify and characterize changes in miRNAs of adipose tissue.

One of the most important result we had was a significant reduction of inflammation biomarkers in hypothalamus. In order to amplify the acknowledgment on how ghrelin affects the whole-body, we also aim to evaluate inflammation biomarkers in other organs like pancreas, liver, BAT and WAT.

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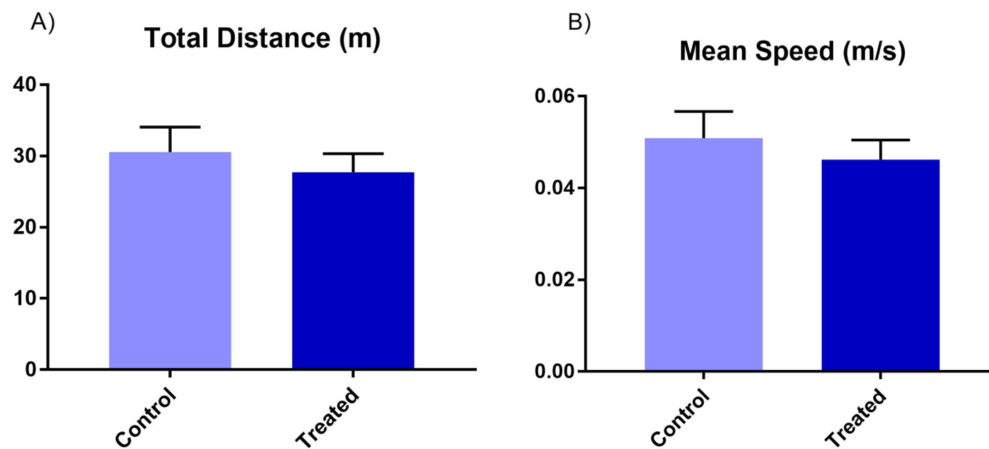
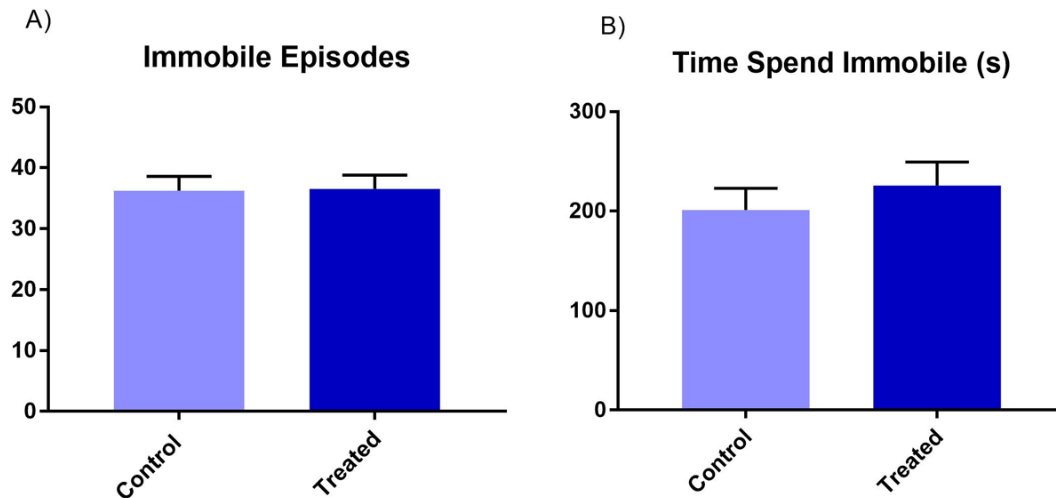
Annexes

Annex 1- *Open field apparatus.*



Annex 2 - *Treated and control animals have similar total distance traveled and mean speed in the open field test.* ***A)*** *Total Distance (m). Control (30.57m ± 3.48m, n=9); Treated (27.73m ± 2.58m, n=12) – P-value = NS (unpaired Student's t-test).* ***B)*** *Mean Speed (m/s). Control (0.051m/s ± 0.006m/s, n=9); Treated (0.046m/s ± 0.004m/s, n=12) – P-value=NS (unpaired Student's t-test). Data were represented as mean ± SEM.*



__Annex 3__ - Treated and control animals have similar immobile episodes and time spend immobile in the open field test. __A)__ Immobile Episodes. [control (36.2 ± 2.4, n=9); Treated (36.5 ± 2.3, n=12) – P-value = NS (unpaired Student's t-test)]. __B)__ Time Spend Immobile (s). [control (201.2s ± 21.6s, n=9); Treated (225.5s ± 23.9s, n=12) – P-value=NS (unpaired Student's t-test)]. Data were represented as mean ± SEM.

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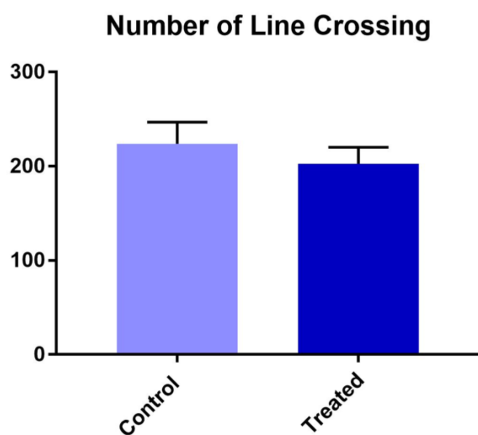
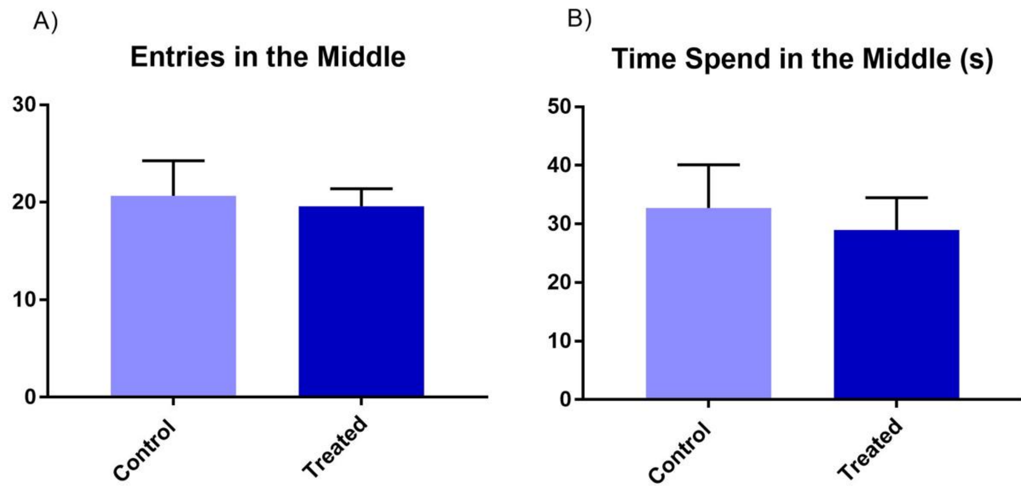
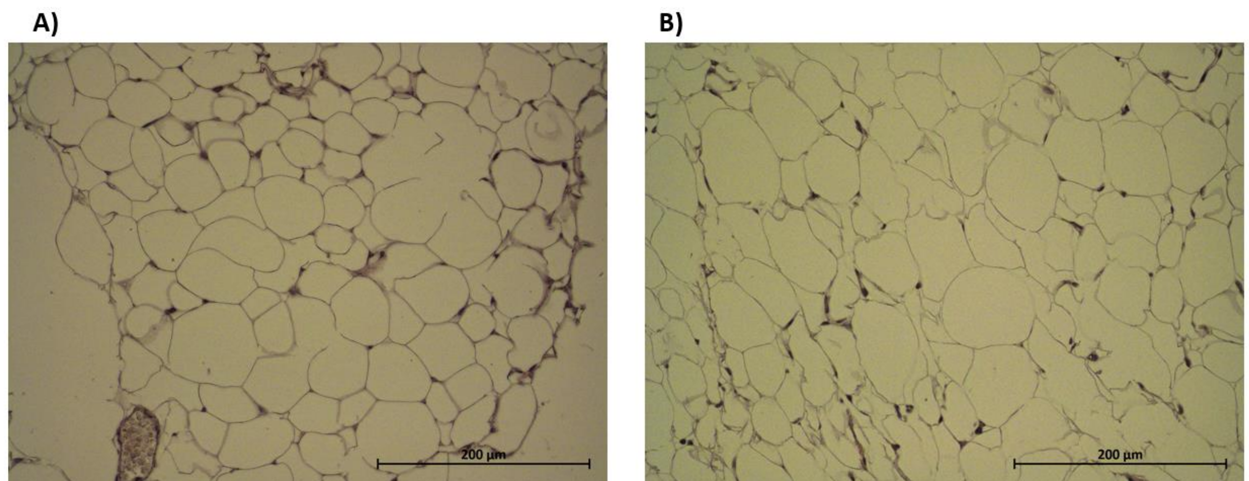


__Annex 4__ - Treated and control animals have similar number of line crossing in the open field test [Control (223.9 ± 23.1, n=9); Treated (202.5 ± 17.7, n=12) – P-value = NS (unpaired Student's t-test)]. Data were represented as mean ± SEM.

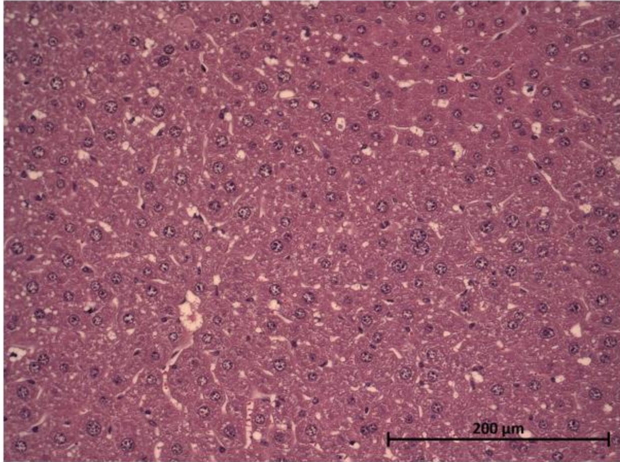


Annex 5 *- Treated and control animals have similar entries in the middle and time spend in the middle in the open field test.* ***A)*** *Entries in the middle. [control (20.67 ± 3.60, n=9); Treated (19.58 ± 1.8, n=12) – P-value = NS (unpaired Student's t-test)].* ***B)*** *Time Spend in the Middle (s). [control (32.72s ± 7.38s, n=9); Treated (28.98s ± 5.52s, n=12) – P-value=NS (unpaired Student's t-test)]. Data were represented as mean ± SEM.*

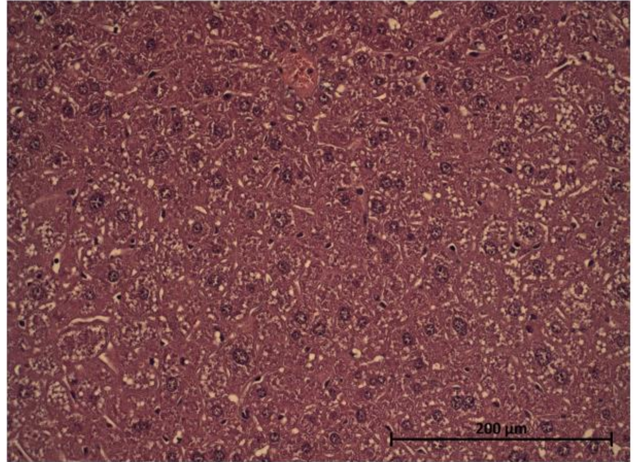


Annex 6 *- Short-term treatment: WAT comparison.* ***A)*** *Control* ***B)*** *Treated.*

A)



B)



Annex 7- *No differences were observed in liver histology.* ***A)*** *Control;* ***B)*** *Treated.*