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Growth hormone-releasing hormone (GHRH) promotes viability and proliferation of neural stem cells and reduces amyloid-β-induced toxicity

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ABSTRACT

Neurogenesis, a process by which new neurons are generated from precursors, persists in discrete regions of the adult hippocampus. The hippocampus is critical for learning and memory and is a main target of Alzheimer's Disease (AD), which causes massive neuronal death, reduction in neurogenesis and impairment in cognitive functions. Therefore, preventing neuronal loss or increasing the production of new neurons may represent a potential therapeutic strategy to reduce AD-induced cognitive decline. Growth hormone-releasing hormone (GHRH), apart from promoting growth hormone (GH) secretion from the pituitary, exerts many extrapituitary functions, including stimulation of cell survival, cardioprotection and protection against diabetic retinopathy. Furthermore, expression of GHRH, as well as GHRH receptor (GHRH-R) and its splice variants (SVs), has been demonstrated in different brain regions, including the cerebral cortex, cerebellum and brain stem cells. To date, however, the role of GHRH on neurogenesis and neuroprotection is still unknown. Thus, we aimed to investigate the role of GHRH on viability, proliferation, apoptosis and differentiation of adult rat hippocampal neural stem cells (NSCs), in stress conditions such as growth factor deprivation and amyloid-β peptide 1-42 ($A\beta_{1-42}$)-induced toxicity, and to define the underlying mechanisms. First, we found expression of pituitary GHRH-R in NSCs. GHRH dose-dependently increased cell viability and proliferation and reduced apoptosis in NSCs cultured under both growth factor deprivation and exposure to $A\beta_{1-42}$; these effects were blocked by the GHRH antagonist JV-1-36. The underlying mechanisms involved $G\alpha_s$/cAMP/PKA/CREB signaling, as demonstrated by results obtained in the presence of specific inhibitors, and phosphorylation of ERK1/2 and PI3K/Akt. In addition, the role of GHRH was examined on differentiation of NSCs into neuronal lineages, such as neurons and astrocytes. Interestingly, GHRH increased mRNA

and protein levels of Tuj1 and GFAP specific marker for neurons and astrocytes, respectively. Moreover, GHRH counteracted the effect of $A\beta_{1-42}$ on elevation of the proapoptotic protein BAX and inhibition of the antiapoptotic protein Bcl-2. Finally, GHRH induced GSK-3β phosphorylation and counteracted the $A\beta_{1-42}$-induced toxicity through inactivation of GSK-3β and inhibition of Tau hyperphosphorylation. Collectively, these results suggest a role for GHRH in preventing neuronal loss and in promoting neurogenesis, with potential therapeutic application of its agonistic analogs in neurodegenerative diseases, such as AD.

INTRODUCTION

1. NEUROGENESIS

Neurogenesis is the process by which new neurons are generated from neural stem cells (NSCs). Since 1944 increasing evidence has been emerging that the adult human brain contains progenitor cells with the potential to produce neuroblasts [1]. However, it was not until 1998 that this fact was confirmed in the adult human brain [2]. It has been clearly demonstrated that the adult mammalian brain retains NSCs that continually generate new neurons within two restricted regions: the subventricular zone (SVZ) of the lateral ventricles [3] and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus [4]. Stem cell self-renewal and progenitor differentiation are regulated by specialized microenvironment, called *niche*, in which these cells reside (Figure 1). The adult neurogenic niche can be seen as a group of cells and local signals that maintain embryonic character to control neurogenesis for life. The neuronal diversity is determined by specific gene expression patterns. The temporal and spatial expression of genes indicate the fate of the neurons, in different regions and functions of the brain [5]. The microenvironment of the niches in addiction to maintaining the population of stem cells, also orchestrates neuronal differentiation. Indeed, NSCs are cells in the adult nervous system that can self-renew and differentiate into all three neural lineages: astrocyte, oligodendrocyte, or neuron. NSCs with the plasticity to give rise to new neurons and glia play a crucial role in the embryogenesis and adult neurogenesis [6]. To become neuron or glia, cells must receive specific cues to activate the specific genes. Various cell surface proteins act as cell markers to identify the different cell lineages and different time-courses [7]. Among these, sex determining region Y-box 2 (Sox2) is an essential transcription factor to maintain self-renewal of undifferentiated embryonic stem cells. Moreover, Sox2 presenting a high expression in embryonic and adult

NSCs during development and is thought to be critical for NSCs proliferation and differentiation [8]. Class III β-tubulin (Tuj1) is a marker of neurons in the central and peripheral nervous systems from the early stage of neural differentiation [7]. While neuronal nuclear protein (NeuN) is used as a neuronal differentiation marker of postmitotic neurons [9]. Glial Fibrillary acidic protein (GFAP) is an intermediate filament-III protein uniquely found in astrocytes and non-myelinating Schwann cells. GFAP gene activation and protein induction appear to play a critical role in astroglia cell activation following central nervous system (CNS) injuries and neurodegeneration [10].

The persistence of hippocampal neurogenesis in the adult brain and its conservation across evolution, in every mammalian species as well as in many non-mammalian vertebrates, suggests that these neurons are relevant for the plasticity of the hippocampus and necessary for biological functions [2,11]. Moreover, several studies demonstrate a correlation between the level of hippocampal neurogenesis and cognition. The regulation of neurogenesis by neural activity suggests that learning and memory might induce the activation of newborn neurons and subsequently enhance their survival and incorporation into circuits [12].

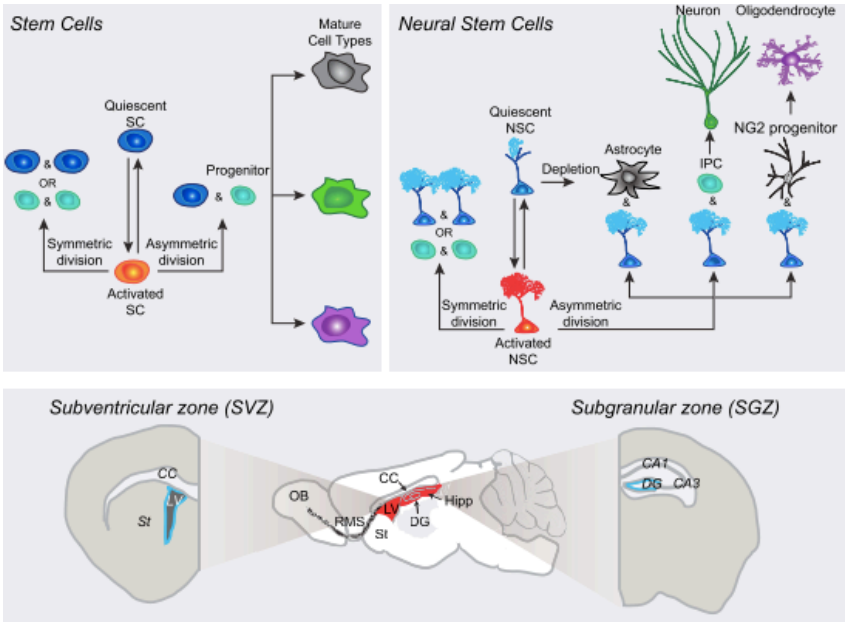

Figure 1. Behavior of Neural Stem Cells within adult niches (Bond A.M., et al., 2015).

1.1 Signaling pathway involved in the regulation of neurogenesis

The activities of immature newborn neurons from both SVZ and SGZ are critical for their survival, differentiation, and subsequent integration into the existing neural circuits. It has been demonstrated that proliferation of NSCs increases both *in vivo* and *in vitro* in response to epidermal growth factor (EGF), fibroblast growth factor-2 (b-FGF), brain-derived neurotrophic factor (BDNF), heparin-binding epidermal growth factor (HB-EGF) and vascular endothelial growth factor (VEGF) [11,12]. Numerous extrinsic factors and intracellular pathways have been implicated in regulating adult SGZ hippocampal neurogenesis. BDNF is a key factor in neurogenesis regulation. The expression levels of BDNF and neurogenesis are positively correlated [13]. These data suggest a role for BDNF signaling in enhancing the survival of newborn neurons. Several signaling molecules regulate hippocampal neurogenesis including Wingless-related integration site (Wnt)/β-catenin, Notch, sonic hedgehog (Shh), bone morphogenetic proteins (BMP), mTOR, growth and neurotrophic factors and neurotransmitters [14,15]. During early neurogenesis the Wnt pathway promotes self-renewal and maintains neural progenitors. Wnt activates the protein Dishevelled (Dvl) by phosphorylation and triggers the stabilization of cytoplasmic β-catenin, that inhibits glycogen synthase kinase 3 β (GSK-3β) activity. β-catenin enters the nucleus and activates the transcription by displacing the repression of Wnt target genes. It has been demonstrated that treatment with lithium, one of GSK-3β inhibitor, promotes rat adult hippocampal NSCs proliferation and neuronal differentiation [16]. In addition to the key roles of the Wnt pathway during development, it has proved to be important in the adult brain, where it regulates synapse formation, neurotransmission, plasticity and neurogenesis [17]. PI3K/Akt is another pathway involved in regulating survival and proliferation of adult hippocampal progenitor [18] and PI3K/Akt-mediated inactivation of GSK-3β through phosphorylation on Ser9 residue is essential for hippocampal neurogenesis and neuronal survival [17,19]. Moreover, it has been demonstrated that PI3K/Akt/GSK-3β/Wnt pathway promotes hippocampal neurogenesis in

a mouse model of Alzheimer's disease (AD) [19,20]. The transcription factor cAMP response element binding protein (CREB) signaling is another central pathway in adult hippocampal neurogenesis, regulating the morphological development and survival of new hippocampal neurons [18,21,22]. The maintenance of expression of proteins involved in neuronal development and the control of neurogenic transcriptional programs are associated with CREB signaling activation [22]. It has been demonstrated that cAMP signaling controls several aspects of neuronal physiology, such as cell survival and proliferation, neurite outgrowth and differentiation through a CREB-independent pathway or through activation of CREB signaling [21]. CREB is usually activated by cAMP-dependent protein kinase (PKA)-induced Ser133 phosphorylation. However, several other kinases, including extracellular signal-regulated kinase (ERK)1/2, protein kinase C (PKC), and p38 mitogen-activated protein kinase (MAPK), regulate CREB activity via phosphorylation, as well [23]. Indeed, ERK1/2 pathway promotes neurogenesis [24], plays a critical role in neuronal cell development in the hippocampus and promotes progenitor cell differentiation [25].

The potential to regulate human neurogenesis should prove to be an interesting area of investigation. However, our knowledge is still limited in terms of the molecular mechanisms underlying cell proliferation, differentiation, survival, and integration during adult neurogenesis.

2. ALZHEIMER'S DISEASE

During aging a modest decline in neurogenesis occurs in human hippocampus, although there is substantial neurogenesis throughout life. Aging brain might be caused by loss of stem cell turnover and by decrease of NSCs proliferation that is known to correlate with a progressive and extensive decline in the physical and cognitive performance. The reduction of adult neurogenesis resulting from neurodegenerative diseases is likely to be caused by selective death of certain neurons and inflammation in diseased brains [14].

Alzheimer's disease (AD) is a neurodegenerative disease characterized by symptoms such as loss of memory and language problems, which tend to become more severe in the course of the disease up to the point in which they interfere with the patient's ability to perform daily routine [26]. Mouse models of AD indicate that the level of neurogenesis is dramatically reduced in the early stage of the disease [27]; proliferation and survival of newborn cells are significantly diminished during its progression. AD is a devastating neurodegenerative disorder with a relentless progression. AD is thought to be caused by a multitude of environmental factors whereas aging represents the greatest risk factor [28]. The neuropathological hallmarks of AD include extracellular deposition of amyloid-β peptide (Aβ) and hyperphosphorylation of Tau, the earlier events in the formation of neurofibrillary tangles (NFTs) [26,29].

2.1 Amyloid-β peptide

The neurotoxic potential of the Aβ results from its biochemical properties that favor aggregation into insoluble oligomers and protofibrils [30]. Among several Aβ isoforms that bear subtle differences depending on the number of C-terminal amino acids, Aβ fragments (1-42) play a pivotal role in the pathogenesis of AD. Soluble Aβ fragments, which are due to overproduction of Aβ and/or failure of clearance mechanisms, are the primary pathogenic factor leading to cognitive impairment in AD [30]. An imbalance between production, clearance, and aggregation of peptides, causes the accumulation of Aβ and this excess may be the initiating factor in AD. This theory is called the "amyloid hypothesis" [31].

Aβ is generated by the proteolytic cleavage of the amyloid precursor protein (APP), a transmembrane protein of uncertain function, possibly involved in the synaptic plasticity [32]. APP undergoes proteolytic cleavage through two mechanisms. The first mechanism, called *non-amyloidogenic,* involves a first cleavage by the α-secretase enzyme, that determines the generation of a soluble secreted fragment, and 83 amino acids long fragment defined sAPPα,

and C83 fragment, respectively. C83 fragment remains bound to the cell membrane and then undergoes a further proteolytic cleavage by the γ-secretase enzyme that produces the p3 fragment, also secreted in the extracellular environment. After these two consecutive cuts, the intracellular domain of APP, called amyloid intracellular domain (AICD), remains in the cell membrane [31,33]. The second cleavage mechanism of APP, defined as *amyloidogenic* process is initiated by β-secretase β-site amyloid precursor protein–cleaving enzyme 1 (BACE-1) that cleaves a membrane-anchored, releasing a APPβ. The retained 99-residue carboxy-terminal fragment (C99) is a γ-secretase substrate, generating Aβ and AICD. γ-secretase cleavage occurs within the cell membrane in unique process termed "regulated intramembranous proteolysis" [31,33] (Figure 2). AICD is a short tail (approximately 50 amino acids) that is released into the cytoplasm after progressive cleavages by γ-secretase. AICD is targeted to the nucleus and activates transcription whereas soluble Aβ is prone to aggregation [30]. Aβ fragments and plaques are potent neurotoxic, block proteasome function, inhibit mitochondrial activity, alter intracellular Ca^{2+} levels and stimulate inflammatory processes. Loss of the normal physiological functions of Aβ is also thought to contribute to neuronal dysfunction. Among several Aβ isoforms, characterized by a different number of amino acids, the $A\beta_{1-42}$ fragment plays a pivotal role in the pathogenesis of AD. In fact, this is the most amyloidogenic fragment due to its β sheet folding that is poorly degradable and that determines the generation of fibrils and subsequently of the neural plaques [29,34].

2.2 Tau protein

Tau is an intracellular protein with multiple functions. It is thought to have a role in binding microtubules, axonal transport, signaling pathways modulation, and neurogenesis in adults. In physiological conditions Tau protein is present in neurons associated with microtubules but post-translational modifications, such as phosphorylation, may lead to accumulation of NFTs and toxic species of soluble Tau [29,34]. Often, these insoluble aggregates of Tau protein

are complexed with ubiquitin which represents the attempt by neurons to degrade the aggregates. The lost association between the Tau protein and microtubules impairs the stability of microtubules and this leads to alterations in all the functions carried out by microtubules, including the axoplasmic transport, the transport of proteins from the soma of the neuron to the axon [35] (Figure 2). Studies have shown that the density of NFTs is directly correlated with the alterations of the neurons and thus with the progressive worsening of dementia [36].

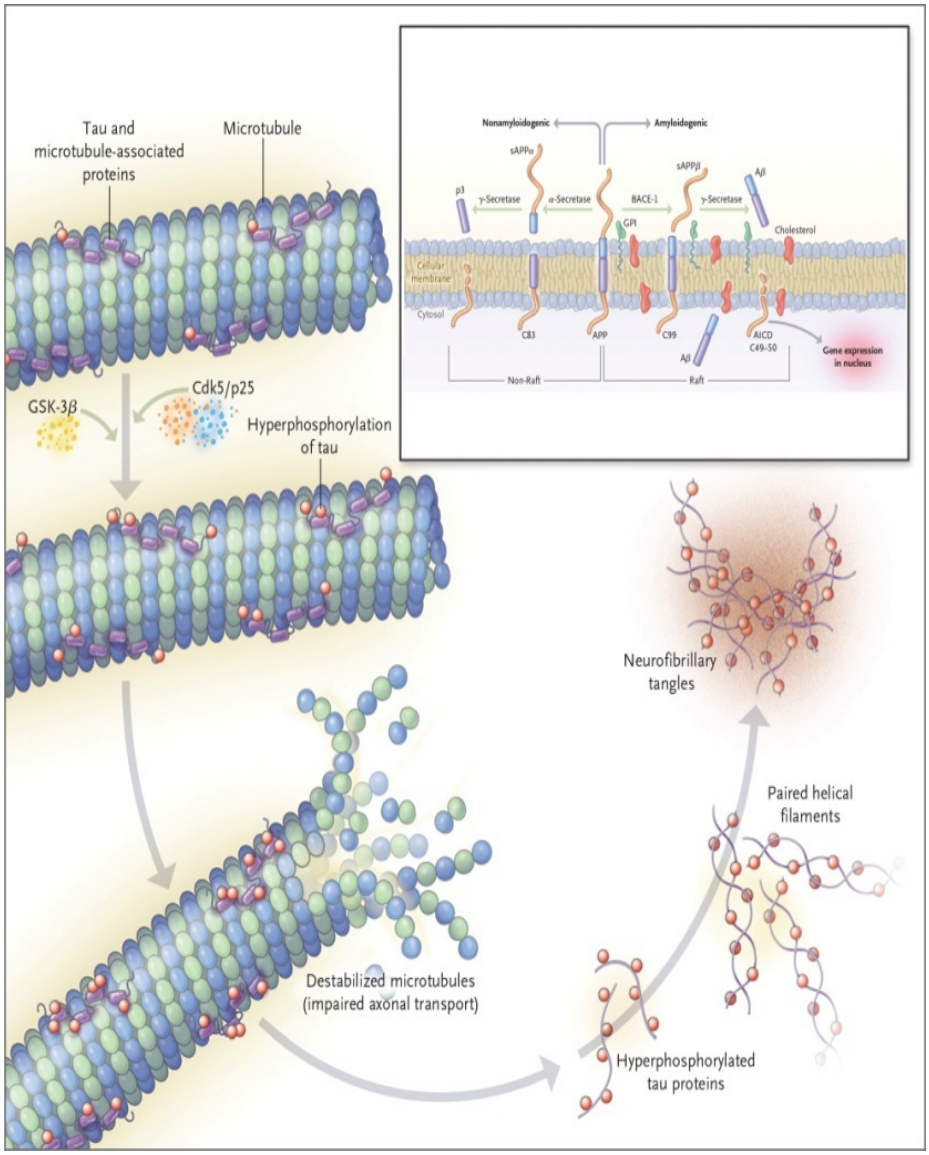

Figure 2. Etiopathology of Alzheimer's disease (Adapted by Querfurth H.W., et al., 2010)

2.3 Alzheimer's disease and neurogenesis

Aβ interacts with the signaling pathways that regulate the phosphorylation of the microtubule-associated protein Tau [29]. Aβ fragments indirectly affect the activity of phosphatases and kinases, in particular GSK-3β [19,37]. In concert, they orchestrate an increasing phosphorylation of protein Tau typical of AD etiopathology. In this manner, GSK-3β activity contributes both to Aβ fragments production and Aβ-mediated neuronal death. Over production of Aβ fragments and the consequent appearance of amyloid plaques cause an overall reduction in the number of adult-generate hippocampal neurons, compromising the replacement in the adult hippocampus [32].

The physiological relation between Aβ and Tau is not clear in normal brain, while their pathological relation is evident in AD brain, although complicated and still largely to be clarified. These two proteins and their associated signaling pathways therefore represent important therapeutic targets for AD [34]. Furthermore, in attempt to identify early diagnostic and prognostic markers for this disease, the release of many neurotrophic factors was found to be altered. It was recently reported that BDNF fluctuated according to AD severity and correlated with loss of cognitive function, suggesting that BDNF plays a role in synaptic loss and cellular dysfunction underlying cognitive impairment in AD [13].

As AD is a neurodegenerative disorder, early symptoms are often mistakenly thought to be "age-related". In the early stages, the most common symptom is short memory loss. As the disease advances long-term memory loss occurs as one of visible markers of CNS profound alteration that involved different brain regions. In particular, dentate gyrus of the hippocampus is especially vulnerable to damage at early stages of AD and has been implicated to contribute to the formation of new memory through the birth of new neurons in SGZ area [4]. Furthermore, strong connection between mnemonic functions and increased neurogenesis has been found in the adult human hippocampus, indicating a correlation between the level of hippocampal neurogenesis and cognition [12].

Considering these findings, the production and the plasticity of new neurons may have an important role in some hippocampal functions. Moreover, increased neurogenesis might be able to ameliorate hippocampal dependent learning performance and memory retention in AD [26]. The interrelationship between neurogenesis and hippocampal cognition restoration seems to be a fascinating area of research and a promising starting point for AD therapeutic approach. Therefore, further studies are needed to test whether impaired SGZ neurogenesis can represent a pivotal component for the pathophysiology of AD.

3. GROWTH HORMONE-RELEASING HORMONE (GHRH)

Growth hormone-releasing hormone (GHRH) is a neuropeptide secreted by hypothalamus that regulates the synthesis and secretion of growth hormone (GH) from the anterior pituitary gland [38]. GHRH was first identified in 1961 and then successfully isolated in 1982 from carcinoid and pancreatic tumors causing acromegaly [39,40]. The peptide isolated from the tumors was identical, at the biological and physical-chemical level, to the GHRH present in hypothalamic tissue extracts, but only later it was also identified and characterized in the hypothalamus [41]. GHRH belong to the secretin family of peptides that includes vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), secretin, glucagon, glucagon-like peptides-1 and -2 and gastric inhibitory peptide (GIP) [42]. Evolutionarily, GHRH is relatively well conserved with homologs being identified in rats, chicken and fish [38]. GHRH is a hormone consisting of 44 amino acids; the first 29 are necessary for the release of GH. It is amidated in the C-terminal position in many species, including human, but not in rodents [43]. Human GHRH gene is localized on the long arm of chromosome 20 in humans, on chromosome 2 in mice and on the long arm of chromosome 3 in rats [44]. The gene is organized into 5 exons and encodes for a precursor protein of GHRH (pre-pro-GHRH). Bioactive, mature human GHRH consists of 40–44 amino acids following complex proteolytic processing of a larger pre-pro-GHRH peptide in a multistep process. The shortest sequence of GHRH that possesses full biological activity consists of the 29 N-terminal amino acid residues (1–29) [38] (Figure 3). Structurally, this sequence of GHRH constitutes the core peptide for the development of agonistic and antagonistic analogs of GHRH [45].

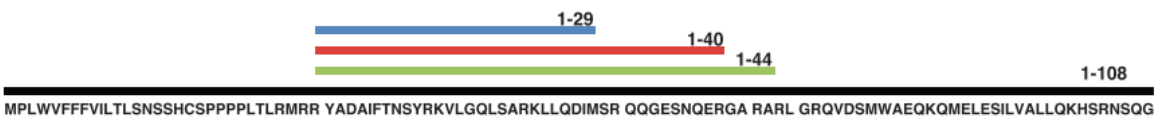

Figure 3. The amino acid (aa) sequence of various forms of GHRH. Pre-pro-GHRH aa (1–108) (black line), the biologically active forms corresponding to aa(1–44) (green line) and aa (1–40) (red line), and the fragment with full biological activity aa(1–29) (blue line) (Kiaris H., et al., 2011).

3.1 GHRH receptor

Pituitary GHRH receptor (GHRH-R) is a transmembrane 7-domain receptor coupled to $G\alpha_s$ protein that activates the adenylate cyclase (AC)/cAMP/protein kinase A (PKA) pathway. Activation of the G-protein complex stimulates AC, which results in the conversion of ATP to cAMP. The production of cAMP induces an increase of intracellular Ca^{2+} that causes the release of GH, stored in the secretory granules. Moreover, the increase of cAMP stimulates the PKA which phosphorylates several transcription factors including the cAMP response element-binding protein (CREB) [46]. GHRH-R, predominantly expressed in the pituitary, has been demonstrated to be also present in many peripheral tissues and cell types, including pancreatic β-cells, myocardium, skeletal muscle cells and fibroblasts [45,47–49] but also in cerebral cortex, cerebellum, and brain stem cells [50]. Similarly, GHRH mRNA was found in several normal tissues including hypothalamus, heart, placenta, the male and female reproductive system, lymphocytes and peritoneal bone marrow-derived cells [50,51]. Recent studies show that GHRH-R and its alternatively spliced variants (SVs) are also expressed in different types of tumors, such as pancreatic carcinoma, kidney, ovary, breast, lung, prostate, and bone [52]. SV1 and SV2 show a great similarity to the pituitary GHRH-R; in particular, they differ in the first three exons that encode for a part of the extracellular domain of the protein; indeed, SV1 loses the region from the first to the third exon, maintaining the third intron, which contains a new in-frame start codon [53].

3.2 GHRH/GH/IGF-1 axis

GH is synthesized, stored and secreted in a pulsatile manner by the somatotropic cells of the anterior pituitary gland, and its release is regulated by three hypothalamic hormones [40,46]. The first is GHRH, which stimulates GH synthesis and secretion by binding to its receptor (GHRH-R). The second hormone is ghrelin, a peptide hormone mainly synthesized at the gastric level but also present in other tissues, including the hypothalamus, which stimulates

the synthesis and secretion of GH binding to specific receptors [40]. Finally, the third hormone is somatostatin (SS) which, by activating specific receptors on the pituitary gland, inhibits GH secretion, but not its synthesis [40]. Most of the biological effects of GH are indirect and mediated by insulin-like growth factor-1 (IGF-1), also called somatomedin C, hormone similar to insulin, which plays an important role in childhood growth and has anabolic effects in adults. Once produced, IGF-1 is released into the circulation where it binds proteins called IGF-binding proteins (IGFBPs) able to increase its plasma half-life [40]. IGF-1 has an insulin-like activity and promotes cell survival, differentiation and maturation of different tissues including bone, cartilage, and skeletal muscle (Figure 4). The biological functions of IGF-1 are performed both via autocrine and paracrine mechanisms [46]. Furthermore, GH secretion is regulated by negative feedback mechanisms by IGF-1 and GH, which can act directly on the pituitary gland by inhibiting GH secretion, or on the hypothalamus by inhibiting the release of GHRH and stimulating the production of SS.

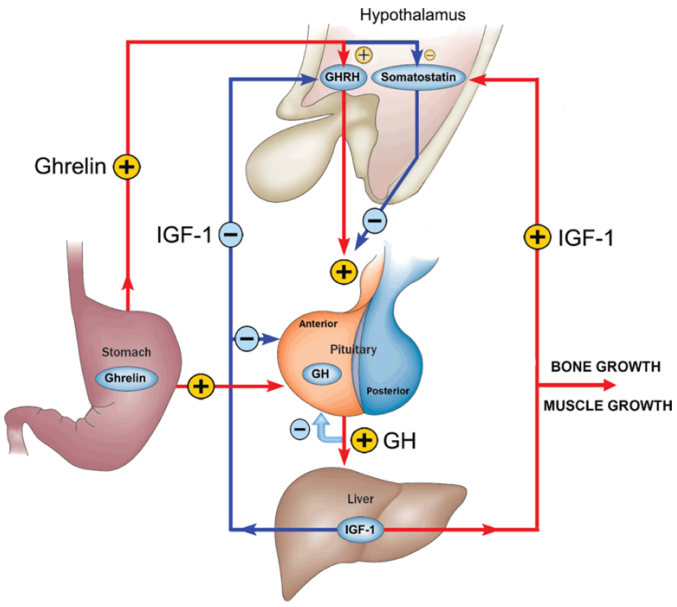

Figure 4. GHRH-GH-IGF-1 axis. (Rivier J., et al., 1982)

3.3 Peripheral effects of GHRH

In the past, GHRH was considered a simple regulator of the GH/IGF-1 axis. However, it is now known that, in addition to endocrine actions, GHRH and its analogs also act in

peripheral organs. The prosurvival activity of GHRH, exemplified by stimulation of the proliferation of various cell types, by the protection of the cells against apoptosis, or both these effects, appears to represent the common function of GHRH in a wide range of peripheral tissues [42,45,52,54]. Therefore, GHRH is expressed in many extra-pituitary tissues, including hippocampus, heart, skeletal muscle, placenta, ovary, testis, endometrium, kidney, gastrointestinal tract and in cells derived from bone marrow [50]. GHRH regulates survival and proliferation of β-cells and pancreatic islets; indeed, GHRH analog JI-36 increases proliferation and reduce apoptosis in rat insulinoma cells (INS-1) and improves the engraftment and functionality of pancreatic islet after transplantation in non-obese diabetic mice with severe immunodeficiency [55]. In addition, it has been shown that GHRH is able to stimulate the secretion of insulin from pancreatic islets in rats [56]. GHRH induces cardioprotection promoting survival and inhibiting apoptosis in H9c2 cardiac cells and in cardiomyocytes of adult rats [47,57]. Moreover, GHRH attenuates cardiac hypertrophy *in vitro* and its analog, MR-409, improves heart function *in vivo* [58]. In addition, GHRH was found to regulate cytokine release from human peripheral blood mononuclear cells (PBMCs) [59], to promote the differentiation of granulocytes from progenitor bone marrow cells into functional mature immune cells [60], to reduce pulmonary edema [61] and the visceral fat, improving dyslipidemia and decreasing the markers of cardiovascular risk [62]. Furthermore, GHRH promotes wound healing [63], reduces ocular inflammation [64], induces survival and prevents apoptosis of C2C12 myotubes treated with tumor necrosis factor-alpha (TNF-α) [48] (Figure 5).

However, GHRH expression has been demonstrated in several cancer cell types, suggesting its involvement in the pathogenesis of cancers and tumor growth by autocrine and/or paracrine mechanisms [65,66]. Indeed, several studies, *in vitro* and *in vivo*, have shown that GHRH antagonists are able to inhibit cell proliferation and survival of cancer cell lines and in mouse xenograft models [67].

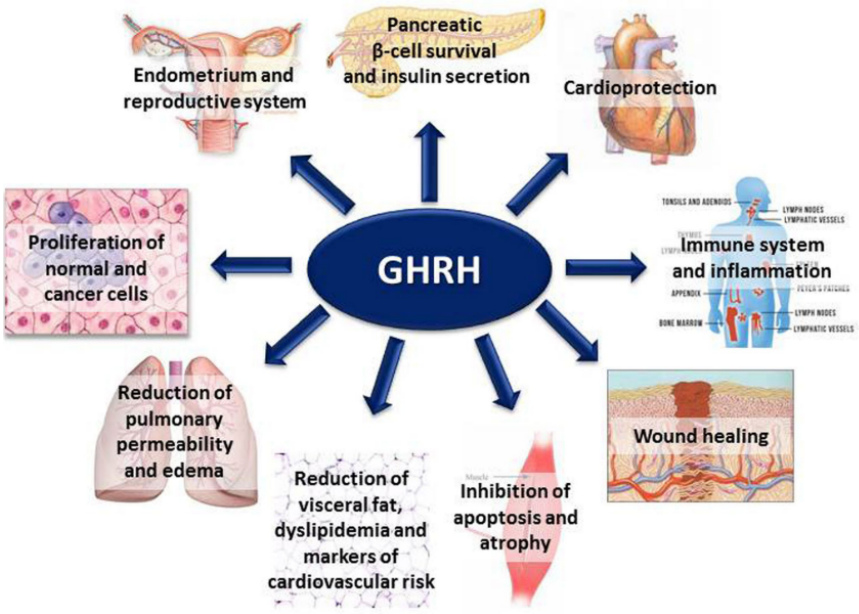

Figure 5. Peripheral effects of GHRH (Granata, R., 2016)

3.4 GHRH analogs

The discovery of the numerous peripheral effects of GHRH in recent years has spurred the development of several synthetic neuropeptide analogs. The synthetic GHRH agonists have been synthesized starting from the shortest GHRH sequence, capable of carrying out the complete biological activity of the peptide and consisting of 29 N-terminal amino acid residues [GHRH (1-29) NH_2] [38]. These molecules, as well as the endogenous neuropeptide, have affinity for GHRH-R and its splicing variants, the characteristic that distinguishes them most is their high stability, especially *in vivo* [52]. In addition to their documented ability to induce GH secretion, GHRH agonists influence peripheral tissues by direct binding to their receptor, stimulating cell proliferation and survival, and inhibiting apoptosis [38,42,54]. The first agonists synthesized were JI-22, JI-34, JI-36 and JI-38 [68]. New analogues of GHRH (MR-356, MR-361, MR-367) were subsequently produced and it has been shown that they have, both *in vitro* and *in vivo*, a greater efficacy in inducing GH release. Finally, the analogues MR-403, MR-406 and MR-409 have been synthesized, which show even greater endocrine activity. Given the protective effects of endogenous GHRH on various peripheral tissues, many studies have evaluated the action of the most powerful GHRH agonists to identify

possible clinical applications. For example, the subcutaneous administration of MR-409 promotes cardioprotective mechanisms in pig models of cardiomyopathy [69], counteracts cardiac hypertrophy *in vitro* and restores cardiac function on *in vivo* models of cardiac hypertrophy [58]. Furthermore, protective effects were also found in mouse β-cell lines, where the agonists MR-356 and MR-409 were able to stimulate survival and cell proliferation [55].

GHRH antagonists are hormone analogues that act as competitors for binding to GHRH-R and its splice variants, causing blocking of receptor signal transduction [67]. For this reason, therapeutic strategies based on the use of GHRH antagonists have been developed for the treatment of various pathological conditions [64,70–72]. The first synthetic peptides produced, (MZ and JV), in addition to having a high affinity for GHRH-R, can inhibit the release of GH both *in vivo* and *in vitro* [73]. In recent years, with the aim of producing peptides with increasing affinity and half-life, a new generation of GHRH antagonists, called MIA, has been synthesized [74]. These new synthetic peptides, in fact, have a higher binding affinity with the receptor, lower endocrine effects on the GH-IGF-1 axis and a high antitumor activity [74]. Therefore, several studies have demonstrated the ability of these molecules to inhibit the growth of different types of tumors such as osteosarcomas, pituitary adenoma, renal, pancreatic, breast, prostate, lung, ovarian, mesothelioma and colorectal cancer [38,65–67]. The action of GHRH antagonists on different types of cancer is also supported by studies demonstrating their ability, *in vitro* and *in vivo*, to inhibit proliferation in different tumor cell lines and xenograft [67].

3.5 GHRH effects on central nervous system

The etiopathogenetic role of the GHRH-GH-IGF-1 system in neurodegenerative disorder, as senile dementia, is still debated [75,76]. Indeed, some studies described a pivotal role of IGF-1 deficiency in the pathogenesis of different neurodegenerative disorders, including AD. Moreover, IGF-1 has numerous positive effects on neurobiological processes compromised

by aging and AD [77,78] with potent neurotrophic and neuroprotective actions including the stimulation of neurite outgrowth, the promotion of neuronal survival in the hippocampus, the regulation of tau phosphorylation [79] and protection from the neurotoxic effects of Aβ [80]. In transgenic mouse models of AD, circulating levels of IGF-1 are low, and when IGF-1 levels are restored in these animals, Aβ is reduced [81]. Finally, in patients with moderate to severe AD, IGF-1 levels are reduced relative to age-matched cognitively normal adults [82]. Conversely, for other authors, inhibition of the different levels of the GHRH-GH-IGF-1 axis apparently exerts a beneficial impact on the progress of Alzheimer's disease [70,76,83]. This phenomenon, however, should not be confused with the effect of these mediators on cognitive performance, *per se*. Concerning Alzheimer's disease, literature data suggest that the inhibition of the axis can be desirable, at least, in the developing disorder [70,83,84]. Data regarding the role of GHRH in CNS are also controversial. Both the agonists and antagonists of GHRH show important effects on CNS, and GHRH-R is present in the brain cortex and other brain areas [50]. In cell cultures, the GHRH analog showed antioxidative and neuroprotective properties; it has been demonstrated that GHRH agonist tesamorelin improves cognitive function in adults with mild cognitive impairment and in healthy older adults [85]. Conversely, exogenous GHRH has been demonstrated to impair hippocampal memory consolidation [86] while antagonists of GHRH exhibited the capability to inhibit amyloid aggregation and decreased the deterioration in cognitive performance in a transgenic mouse model of AD [42,54,84]. Furthermore, it has been demonstrated that GHRH play a key role in sleep regulation [87]; indeed, it regulates sleep mechanisms in AD mice experimental models [88] and promotes sleep in male patients with depression [89]. Moreover, GHRH agonist JI-34 attenuates hypoxia-induced neurocognitive deficits, anxiety, and depression in mice [90]. However, GHRH biological activities in the hippocampus and its effects on neurogenesis and neuroprotection are yet to be explored.

AIM OF THE STUDY

It has been clearly demonstrated that the adult mammalian brain retains neural stem cells that continually generate new neurons. It is becoming increasingly clear that adult neurogenesis readily responds to several conditions and stimuli, such as growth factors or hormones, which directly induce the generation of new neurons or indirectly promote neuronal survival, proliferation and activation of antiapoptotic pathways [91–93]. However, neurogenesis deregulation is also correlated with neurodegenerative diseases, such as AD, an age-related neurodegenerative disorder characterized by impairment of memory and cognitive function [32]. Indeed, several studies indicate that the level of neurogenesis is dramatically reduced in the early stage of AD, and proliferation and survival of newborn cells are significantly diminished during its progression [94].

In addition to its endocrine role, GHRH exerts a wide range of extrapituitary effects which include stimulation of cell survival, proliferation and differentiation and inhibition of apoptosis in several cell types and tissues [38,42,52]. Furthermore, it has been demonstrated that, at the level of CNS, GHRH regulates sleep mechanisms in AD mice experimental models [88] and attenuates hypoxia-induced neurocognitive deficits, anxiety, and depression in mice [90], whereas biological activities in the hippocampus and its effects on neurogenesis and neuroprotection are yet to be explored.

Thus, the aim of this study was to clarify the role of GHRH on viability, proliferation, apoptosis and differentiation of adult rat hippocampal neural stem cells (NSCs) in stress conditions such as growth factor deprivation and amyloid-β peptide 1-42 ($A\beta_{1-42}$)-induced toxicity. Moreover, the underlying signaling pathways were explored, specifically those related to neurogenesis and neuronal cell survival and differentiation.

First, the protective role of GHRH was studied with regards to the involvement of GHRH receptor (GHRH-R) signaling and activation of cAMP/PKA/CREB pathway. Furthermore, the involvement of ERK1/2 and PI3K/Akt pathways was assessed on the mitogenic effects of GHRH. In addition, it has been investigated whether GHRH increased the expression of specific markers of differentiation in NSCs. Finally, the study tried to understand how GHRH modulates intracellular Aβ-induced alteration, such as GSK-3β variation and Tau abnormal phosphorylation.

In conclusion, a greater understanding of the mechanisms involved in the progression of AD and into the protective role of GHRH is essential for preventing neuronal loss and promoting neurogenesis, as well as for the developing new potential therapeutic strategies for GHRH analogs in neurodegenerative diseases, such as AD.

MATERIALS & METHODS

Reagents

Rat GHRH (1-44)-NH2 was from MyBiosource (San Diego, USA). Amyloid-β protein fragment (1-42) and JV-1-36 were from Phoenix Pharmaceuticals (Belmont, CA). NF449, MDL12330A, KT5720, PD98059, wortmannin, 2,5-diphenyl tetrazolium bromide (MTT), Dulbecco′s Modified Eagle′s Medium (DMEM)/ F12, fetal bovine serum (FBS), bovine serum albumin (BSA), penicillin, streptomycin, amphotericin B, L-glutamine, 3-isobutyl-1-methylxanthine (IBMX), polyornithine, primers and cell culture reagents were from Sigma-Aldrich (Milan, Italy). Human b-FGF (fibroblast growth factor), B27 supplement and N2 supplement were from Life Technologies, Inc. (Invitrogen, Milan, Italy). Mouse monoclonal antibody for β-actin (sc-376421), rabbit polyclonal antibody for Bcl-2 (sc-783) and goat polyclonal antibody for p-Tau (Ser396) (sc-12414) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal antibodies for GHRH-R (ab76263), NeuN (ab177487) and GFAP (ab7260) were obtained from Abcam (Cambridge, UK). Rabbit polyclonal antibodies P-CREB (Ser133) (code 9191S), P-ERK1/2 (Thr202/Tyr204) (code 9101S), P-Akt (Ser473) (code 4060), P-GSK-3b (Ser 9) (code 9336), P-p70S6K (Thr389) (code 9205), Bax (code 5023S) were from Cell Signaling Technology (Euroclone, Milan, Italy). RT-PCR and Real-Time PCR reagents were obtained from Life Technologies, Inc. (Invitrogen, Milan, Italy).

Cell culture and differentiation

Adult Rat Hippocampal Neural Stem Cells (NSCs) were obtained from Sigma-Aldrich (Milan, Italy). The cells were grown in polyornithine (PORN)-coated flasks or wells and cultured in Dulbecco′s Modified Eagle′s Medium (DMEM)/F12 with B27 Supplement and

b-FGF 20 ng/ml (normal medium, NM) at 37° in a 5% CO_2 humidified atmosphere for normal proliferating conditions. For experimental conditions the cells were switched to DMEM)/F12 supplemented with 0.1% BSA without growth factors and b-FGF for 2 h (control medium, c), then replaced with the same fresh medium, in either absence or presence of the different stimuli for further 24 h. For differentiation conditions, cells were seeded in NM in PORN-coated 6-well plates at a concentration of 50 X 10^4 cells/well. After confluence (d 0), the medium was changed to differentiation medium (DM) [DMEM/F12 with N2 Supplement, 1% FBS and retinoic acid (RA) (1 μM)] for 5 days (d 5) in either absence or presence of GHRH (5 μM).

Cell viability and proliferation

Cells were seeded in NM in PORN-coated 96-well plates at a cell concentration of 8 X 10^3 cells/well. After 48 h, the medium was changed to control medium, and cells were then treated with different stimuli for further 24 h. Cell viability was assessed by MTT assay. Cells were incubated with 1 mg/ml of MTT for approximately 2 h, then the medium was removed, and formazan products solubilized with 100 μl dimethyl sulfoxide (DMSO). Cell viability was assessed by spectrophotometry at 570 nm absorbance using the LT-4000 microplate reader (Euroclone, Milano, Italy). Cell proliferation was assessed using the 5-bromo-2-deoxyuridine (BrdU) incorporation ELISA kit (Roche Diagnostic SpA, Milano, Italy). Briefly, the cells were incubated with BrdU labeling solution for 2 h at 37° C. After removal of the labeling solution, cells were fixed, denatured, and incubated for 90 min with anti-BrdU antibody conjugate, which was subsequently removed by rinsing three times. Finally, cells were incubated in substrate solution at room temperature and proliferation assessed by colorimetric detection at 450 nm absorbance using the LT-4000 microplate reader (Euroclone, Milano, Italy).

Caspase-3 Activity

Cells were seeded in PORN-coated 6-well plates at a concentration of 50 X 10^4 cells/well. Caspase-3 activity was assessed by Caspase-3 Colorimetric Assay Kit (BioVision, USA) in cell lysates, according to the manufacturer's instruction. Briefly, cells were resuspended in Cell Lysis Buffer, incubated 10 min at 4° C, centrifuged and cytosolic extract was used for protein quantification. Next, samples were incubated for 2 h with DEVD-pNA substrate. Caspase-3 activity was assessed by colorimetric detection at 405 nm absorbance with LT-4000 microplate reader (Euroclone, Milano, Italy).

cAMP assay

Cells were seeded in NM in PORN-coated 6-well plates at a concentration of 50 X 10^4. After 48 h, the medium was changed to control medium, and the cells were incubated in the presence of 100 μM of 3-isobutyl-1-methylxanthine (IBMX), either with or without GHRH (1 μM) at the indicated time. Intracellular cAMP was measured from cell lysates using the Cyclic AMP Assay (R&D System, Space Srl, Milan, Italy), according to the manufacturer's instructions.

Western blotting

Protein extraction and Western blot analysis were performed as described previously [65]. Proteins (40 μg) were resolved in 10% SDS-PAGE (12% for Bcl-2 and Bax), transferred to a nitrocellulose membrane and incubated overnight at 4°C with the specific antibodies (dilution 1:500 for Bcl-2, 1:1000 for GHRH-R, P-ERK1/2, P-Akt, P-CREB, P-GSK-3β, P-p70S6K, P-Tau, Bax, and 1:10000 for NeuN and GFAP). Blots were re-probed with β-actin (dilution 1:500) or with the respective total antibodies for protein normalization. Immunoreactive proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit or mouse anti-goat (1:4000) secondary antibodies by enhanced

chemiluminescence substrate (ECL) using ChemiDoc XRS (Bio-Rad, Milan, Italy). Densitometric analysis was performed with Quantity One software (Bio-Rad, Milan, Italy).

Real-Time PCR

Total RNA isolation and reverse transcription (RT) to cDNA (1 µg RNA) from NSCs cells, treated with TRIzol reagent (Life Technologies, Milan, Italy), was performed as described previously [95]. For real-time PCR, reaction was performed with 50 ng cDNA, 100 nm of each primer and IQ-SYBR-green Mastermix (Bio-Rad, Milan, Italy) using the ABI-Prism 7300 (Applied Biosystems, Milan, Italy). The following primer pairs were used: GFAP, forward 5'-CTCAGTACGAGGCAGTGGCC-3', reverse 5'-CGGGAAGCAACGTCTGTGA-3' (NM_017009.2); Tuj1, forward 5'-TAGACCCCAGCGGCAACTAT-3', reverse 5'-GTTCCAGGTTCCAAGTCCACC-3' (NM_139254.2) (designed with the Primer 3 Software, http://www.primer3.org/). 18S rRNA was used as endogenous control. Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Statistical analysis

Results are presented as mean ± SEM. Significance was calculated by two-tailed Student's t-test or one-way ANOVA followed by Dunnet's or Tukey's multiple comparison post-hoc test, as appropriate, using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Significance was established for $P < 0.05$.

RESULTS

GHRH promotes cell viability and proliferation and prevents apoptosis of NSCs through involvement of GHRH receptor signaling

Initially, the protein expression of GHRH receptor (GHRH-R) was assessed in adult rat hippocampal neural stem cells (NSCs) in basal conditions. Moreover, no variation on expression levels of GHRH-R was observed in cells treated with GHRH (5 μM) for 24 h (Figure 1A).

The role of GHRH(1-44)NH_2 was first assessed on viability and proliferation in NSCs cultured in growth factor-deprived medium in presence of increasing concentrations of GHRH (0.5 – 5 μM) for 24 h. As expected, cell viability and proliferation were reduced under growth factor deprivation (control), compared with normal medium (NM). Conversely, GHRH dose-dependently increased cell viability and proliferation as compared with untreated cells (control), showing the strongest effect at 5 μM, that was used for the subsequent experiments (Figure 1B and C). Furthermore, at 24 h GHRH reduced growth factor deprivation-induced apoptosis, as indicated by reduced caspase-3 activity (Figure 1D). Next, to assess the involvement of GHRH-R, NSCs were treated with GHRH for 24 h, in combination with the GHRH-R antagonist JV-1-36 (JV). The increase in cell viability and proliferation induced by GHRH was suppressed by JV-1-36, that had no effects alone, suggesting the involvement of GHRH-R signaling in the protective activities of GHRH (Figure 1E and F). Overall, these results indicated that GHRH exerts survival, proliferative and antiapoptotic effects in NSCs through involvement of GHRH-R signaling.

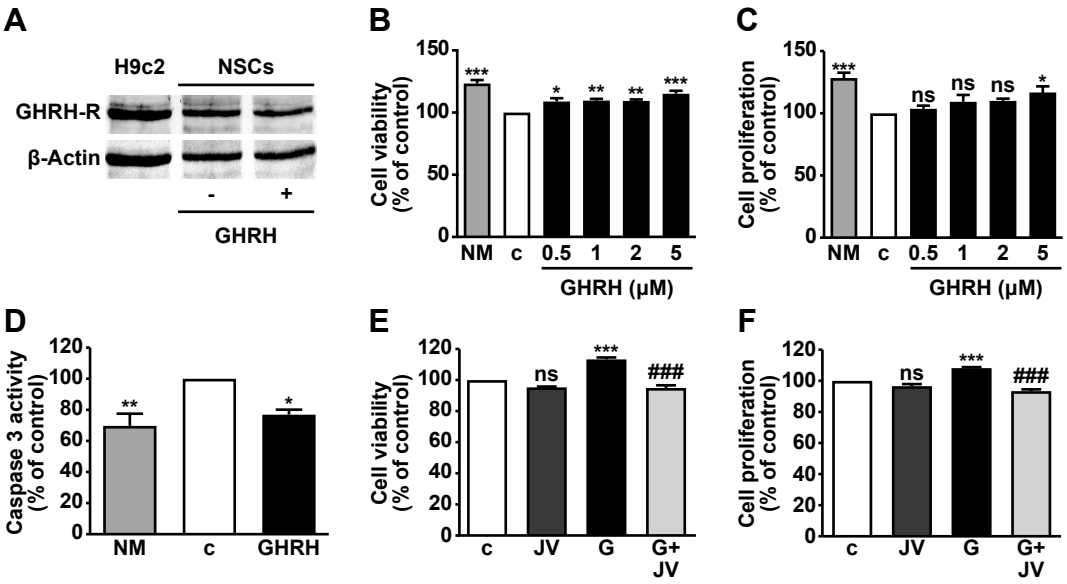

Figure 1. Survival, proliferative and antiapoptotic effects of GHRH in NSCs. (**A**) Protein expression for GHRH-R assessed by Western blot in NSCs cultured in growth factor-deprived medium (control medium, c), in either absence (-) or presence (+) of GHRH (5 μM), for 24 h. H9c2 cardiac cells were used as positive control for GHRH-R and β-actin served as internal control. (**B-C**) Cells were cultured in either normal medium (NM) or in growth factor-deprived medium (c) for 2 h and then for further 24 h with GHRH, at the indicated concentrations. Cell viability (**B**) and proliferation (**C**) were assessed by MTT assay and BrdU incorporation, respectively. (**D**) Apoptosis was evaluated by caspase-3 activity and assessed in NSCs treated for 24 h in control medium (c) with GHRH (5 μM). (**E-F**) Cells were cultured in growth factor-deprived medium (c) for 2 h and then for further 24 h with GHRH (G, 5 μM) or JV-1-36 (JV, 1 nM), either alone or in combination. Cell viability (**E**) and proliferation (**F**) were assessed by MTT assay and BrdU incorporation, respectively. The results are expressed as percentage of control and reported as means ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. c; $^{###}P < 0.001$ vs. G; ns, not significant) (n = 5).

GHRH promotes cell viability and proliferation through activation of $G\alpha_s$/cAMP/PKA signaling and phosphorylation of cAMP response element binding protein (CREB)

Activation of the cAMP/PKA cascade plays a significant role in the regulation of adult neurogenesis [21,96]. Moreover, CREB regulates survival, proliferation, and differentiation of newborn neurons [97] and in the adult brain, it participates in neuronal plasticity, learning, and memory [22]. Therefore, we next investigated whether GHRH-induced effects on cell viability and proliferation would imply activation of the cAMP/PKA/CREB pathway. Our preliminary data suggest that intracellular cAMP levels in NSCs increased at 5 min in response to GHRH (5 μM), then decreased at 15 and 30 min and returned to basal levels at 60 min. The adenylyl cyclase activator forskolin (FK) was used as positive control and strongly increased cAMP levels, as expected (Figure 2A). GHRH also promoted the phosphorylation of the transcription factor CREB on serine 133, which peaked at 30 min,

decreasing thereafter (Figure 2B). Furthermore, inhibitors of the cAMP/PKA pathway were used to clarify the signaling cascade involved in GHRH effects. The $G\alpha_s$ protein-coupled receptor antagonist NF449 (NF), which prevents adenylyl cyclase activation and cAMP production, blocked GHRH mitogenic effects at 24 h (Figure 2C and D). Similar results were obtained by coincubation of NSCs with GHRH and either the adenylyl cyclase inhibitor MDL12330A (MDL) (Fig. 2E and F) or the PKA inhibitor KT5720 (KT) (Fig. 2G and H). Collectively, these findings suggest that GHRH promotes NSCs viability and proliferation through activation of the $G\alpha_s$/cAMP/PKA/CREB pathway.

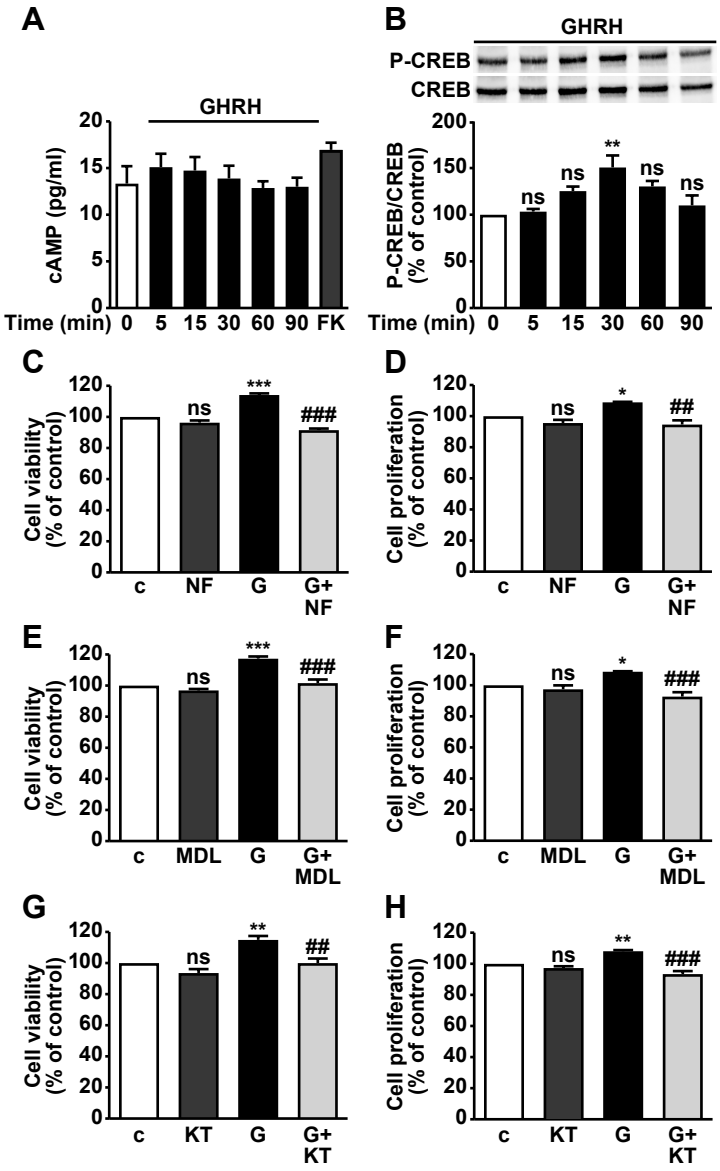

Figure 2. GHRH-induced viability and proliferation involve signaling through $G\alpha_s$/cAMP/PKA and CREB. (**A**) Intracellular cAMP levels in growth factor deprived NSCs cultured for the indicated times with GHRH (5 μM), in the presence of the phosphodiesterase inhibitor IBMX (100 μM), that was added 30 min before stimulation. Forskolin (FK) (50 μM for 2 min) was used as positive control (n = 1). (**B**) CREB

phosphorylation on serine 133 evaluated by Western blot in lysates from NSCs incubated with GHRH (5 μM) for the indicated times. Equal protein loading was determined by reprobing with total CREB antibody. Blots are representative of three independent experiments. Graphs show the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percent of basal ($^{**}P < 0.01$ vs. time 0; ns, not significant). (**C-H**) Cell viability and proliferation assessed by MTT and BrdU, respectively, in NSCs cultured in growth factor-deprived medium (c) for 2 h, and then for further 24 h with NF449 (NF, 25 μM) (**C-D**), MDL12330A (MDL, 100 nM) (**E-F**), or KT5720 (KT,100 nM) (**G-H**), either alone or with GHRH (G, 5 μM). The results are expressed as percentage of control and reported as means ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. c; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.01$ vs. G; ns, not significant) (n = 4).

Phosphorylation of extracellular signal-regulated kinase (ERK1/2), phosphatidylinositol 3-kinase (PI3K)/Akt and glycogen synthase kinase-3β (GSK-3β), but not mTOR, is essential for GHRH mitogenic effects in NSCs

Both mitogen-activated protein kinase (MAPK)/ERK1/2 and PI3K/Akt pathways have been shown to be involved in regulating survival and proliferation of adult hippocampal progenitor [18,98]. Furthermore, PI3K/Akt-mediated inactivation of GSK-3β through phosphorylation on Ser9 residue is crucial for hippocampal neurogenesis and neuronal survival [17]. GHRH, in turn, was found to promote survival and proliferation of different cell types via activation of both ERK1/2 and PI3K/Akt [47,48]. Here, GHRH increased ERK1/2 phosphorylation in NSCs, which peaked at 5 min and decreased thereafter, compared to basal (Figure 3A). Furthermore, GHRH-treated NSCs showed a strongly and time-dependent increase of Akt phosphorylation, which peaked at 30 and 60 min and decreased at 90 min, compared to basal (Figure 3B). Coincubation for 24 h with either the protein kinase kinase (MEK) inhibitor PD98059 (PD), which prevents ERK1/2 phosphorylation, or with the specific PI3K inhibitor wortmannin (W), both abolished GHRH effects on cell viability and proliferation, whereas no effect was observed using the inhibitors alone (Figure 3C-F). Similarly, GHRH strongly and time-dependently increased GSK-3β phosphorylation on Ser9, which peaked at 60 min and decreased at 90 min after stimulation (Figure 3G). Conversely, GHRH had no effect on mTOR downstream target p70S6K (Figure 3H). Overall, these results suggest that GHRH-induced protection in NSCs requires activation of both ERK1/2 and PI3K/Akt, as well as inactivation of GSK-3β, but not mTOR.

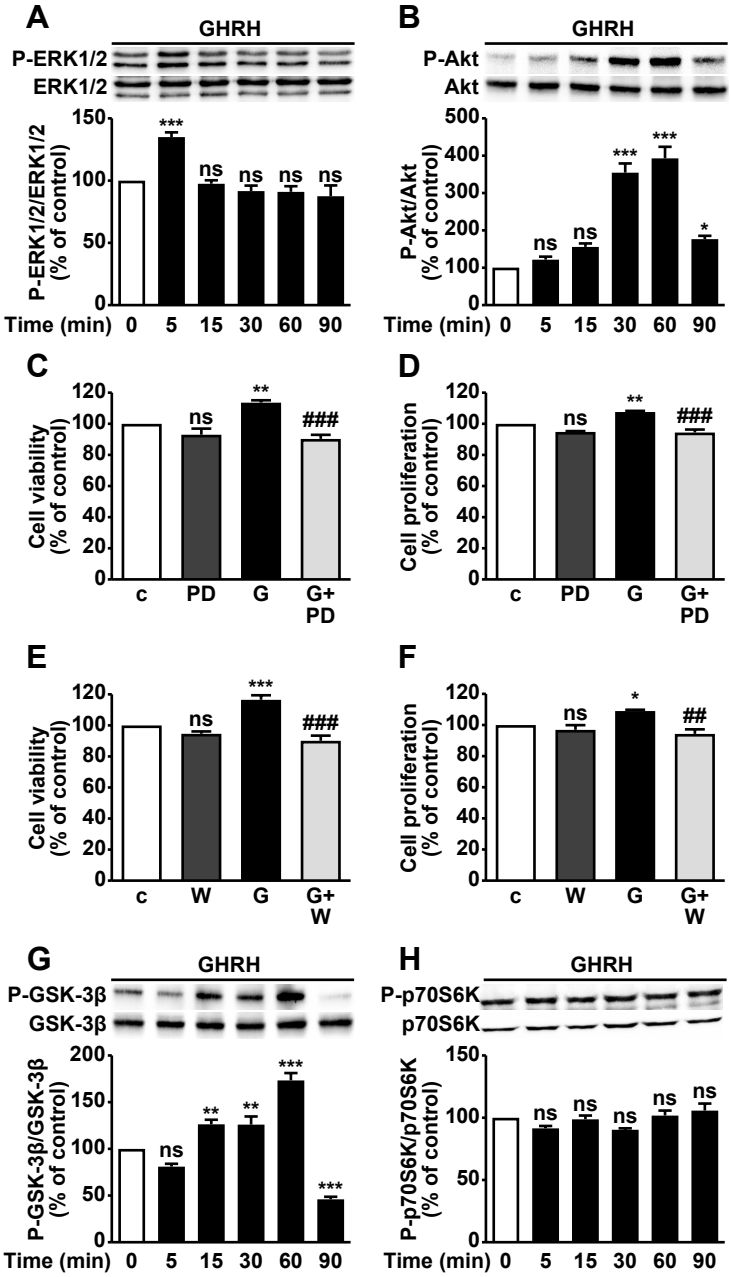

Figure 3. GHRH viability and proliferative effects in NSCs require activation of both ERK1/2 and PI3K/Akt, as well as inactivation of GSK-3β. (**A**) ERK1/2 and (**B**) PI3K/Akt phosphorylation evaluated by Western blot in NSCs incubated with GHRH (5 μM) for the indicated times. Blots were reprobed with total antibodies for normalization. Blots are representative of three independent experiments. Graphs show the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percentage of basal ($^{*}P < 0.05$, $^{***}P < 0.001$, vs. time 0; ns, not significant). (**C-F**) Cell viability and proliferation assessed by MTT and BrdU, respectively, in NSCs cultured in growth factor-deprived medium (c) for 2 h, and then for further 24 h, with GHRH (G, 5 μM) either alone or in combination with PD98059 (PD, 10 μM) (**C-D**) or wortmannin (W, 25 nM) (**E-F**). Data, expressed as percent of control, are the mean ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. c; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. G; ns, not significant) (n=5). **(G)** GSK-3β phosphorylation on Ser9 and (**H**) p70S6K phosphorylation assessed by Western blot in NSCs treated with GHRH (5 μM) for the indicated times. Blots were reprobed with total antibodies for normalization and are representative of three independent experiments. Graphs represent the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percentage of basal ($^{**}P < 0.01$, $^{***}P < 0.001$, vs. time 0; ns, not significant).

GHRH increased the expression of specific markers of differentiation

Neural stem cells (NSCs) are self-renewing cells with the capacity to differentiate into neurons and glia [99]. To investigate the ability of GHRH to induce a major differentiation into neuronal lineages, we examined the expression of specific cell markers for neurons and astrocytes. For differentiation conditions cells were seeded in NM and, after confluence (d 0), cultured in differentiation medium (DM) for 5 days (d 5) in either absence or presence of GHRH (5 μM). The only change of medium was already sufficient to induce differentiation. Interestingly, GHRH increased the mRNA levels of both neuronal marker *Tuj* (neuron-specific class III β-tubulin) and the astrocytes marker *GFAP* (glial fibrillary acidic protein) (Figure 4A and B). Moreover, Western blot analysis showed that GHRH induce a most significant increase of NeuN (neuronal nuclei protein) and GFAP compared with untreated differentiated cells (Figure 4C). Taken together, these data suggest that GHRH mainly increases the differentiation of NSCs into neurons and astrocytes.

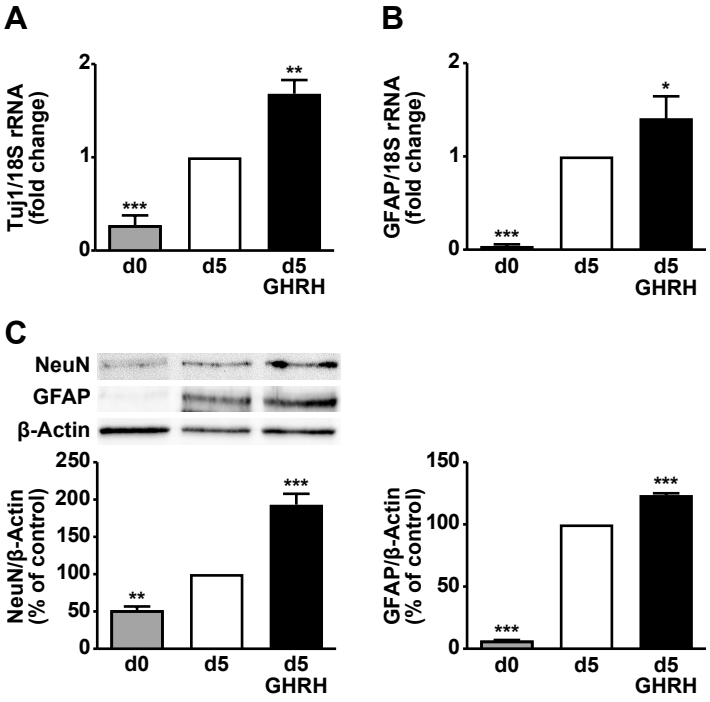

Figure 4. GHRH effects on NSCs differentiation. After confluence (d 0), cells were cultured in differentiation medium (DM) for 5 days (d 5) in either absence or presence of GHRH (5 μM). (**A**) *Tuj1* and (**B**) *GFAP* mRNA evaluated by real-time PCR. Results, normalized to 18S rRNA, are expressed as fold-change of control (d5) and are mean ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. d5) (n = 5). (**C**) NeuN and GFAP assessed by Western blot on lysates from cells stimulated for 5 days with or without GHRH (5 μM). Equal protein loading was determined by reprobing with antibodies to β-actin. Graphs represent the densitometric analysis of proteins normalized to β-actin and reported as percentage of control ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. d5).

GHRH counteracts the detrimental effects of amyloid-β peptide (Aβ)$_{1–42}$

Aβ deposition plays a critical role in the pathogenesis of AD and contributes to neuronal apoptotic cell death in a variety of neural cell types [30]. Moreover, Aβ$_{1-42}$ promotes the formation of neurofibrillary tangles (NFTs), composed of hyperphosphorylated Tau protein [36]. Based on the observed mitogenic effects in growth factor-deprived conditions, we aimed to determine whether GHRH would also protect against Aβ-induced cell death. Cell viability was first assessed in NSCs treated with increasing concentrations of Aβ$_{1-42}$ (0.1 – 2 μM) for 24 h. Aβ$_{1-42}$ reduced cell viability in a dose dependent manner when compared with control (data not shown). The most effective concentration of Aβ$_{1-42}$ in terms of reduction of cell viability was 200 nM that was selected as the best concentration for subsequent experiments. To next investigate the potential protective role of GHRH, the cells were cotreated with Aβ$_{1-42}$ (200 nM) and with increasing concentrations of GHRH (1- 5 μM) for 24 h. GHRH dose-dependently blocked the detrimental effects of Aβ$_{1-42}$ and restored cell viability and proliferation (Figure 5A and B). However, these effects were abolished by addition of the GHRH-R antagonist JV-1-36 (JV) (Figure 5C and D). Moreover, GHRH reduced apoptosis induced by 24 h of treatment with Aβ$_{1-42}$, by reducing caspase-3 activity even below the control levels (Figure 5E). Furthermore, GHRH counteracted the effect of Aβ$_{1-42}$ on elevation of the proapoptotic protein BAX (Figure 5F) and inhibition of the antiapoptotic protein Bcl-2 (Figure 5G). Finally, to clarify the signaling cascades involved in the effects of GHRH, NSCs were treated with Aβ$_{1-42}$ alone or with GHRH for 24 h in combination with specific inhibitors of the cAMP/PKA pathway. Both NF449, MDL12330A and KT5720, that had no effects alone, blocked the protective effects of GHRH against the detrimental effect of Aβ$_{1-42}$ on cell viability and proliferation (Figure 6A-F). Collectively, these findings indicate that GHRH reduces Aβ$_{1-42}$-induced toxicity in NSCs through involvement of GHRH-R signaling and Gα$_s$/cAMP/PKA/CREB pathway.

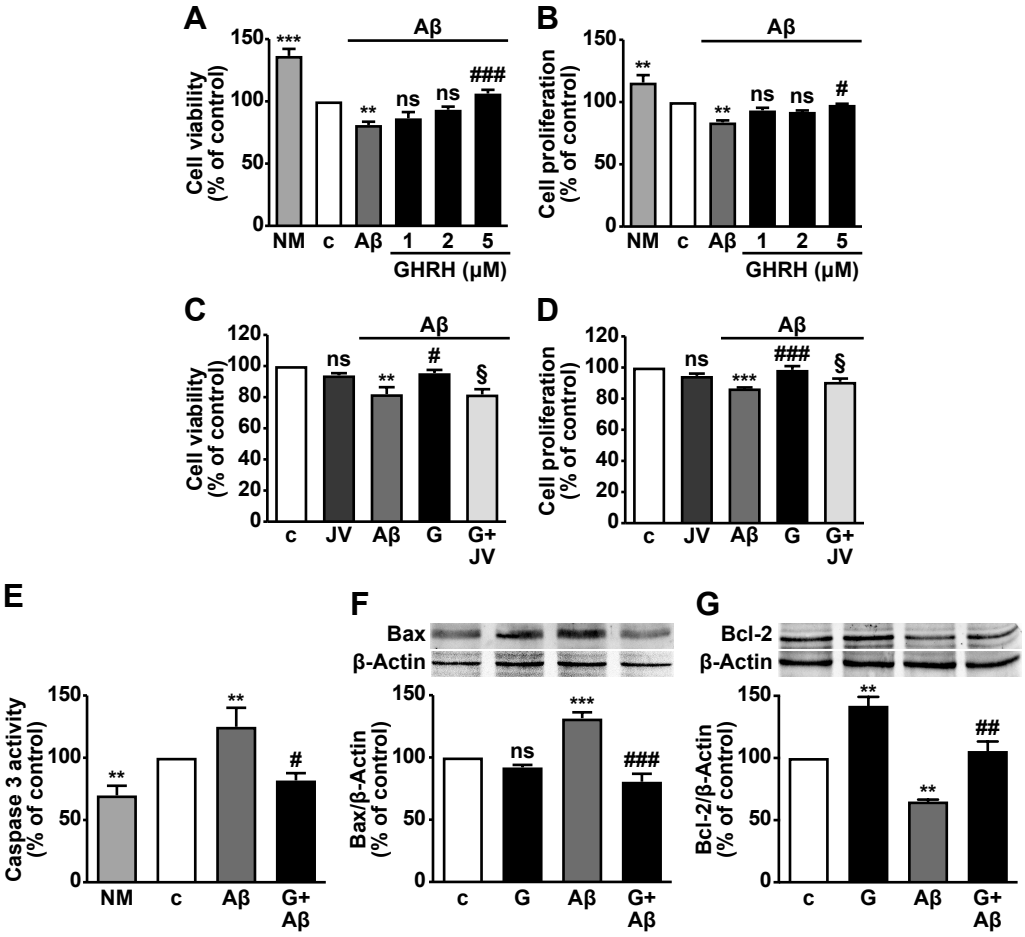

Figure 5. GHRH protective effects against Aβ$_{1-42}$-induced toxicity. Cells were cultured in either normal medium (NM) or in growth factor-deprived medium (c) for 2 h and then for further 24 h with Aβ$_{1-42}$ (0.2 μM), alone or in combination with GHRH at the indicated concentrations. (**A**) Cell viability and (**B**) proliferation were assessed by MTT assay and BrdU incorporation, respectively. The results are expressed as percentage of control and reported as means ± SEM ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. c; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. Aβ; ns, not significant) (n = 5). (**C**-**D**) Cells were cultured in growth factor-deprived medium (c) for 2 h and then for further 24 h with Aβ$_{1-42}$ (0.2 μM) or JV-1-36 (JV, 1 nM), either alone or in combination with GHRH (G, 5 μM). Cell viability (**C**) and proliferation (**D**) were assessed by MTT assay and BrdU incorporation, respectively. The results are expressed as percentage of control and reported as means ± SEM ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. c; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. Aβ, $^{\S}P < 0.05$ vs. Aβ + G; ns, not significant) (n = 5). (**E**) Apoptosis was evaluated by caspase-3 activity and assessed in NSCs cultured in control medium (c) for 24 h, either with or without Aβ$_{1-42}$ (0.2 μM) alone or with GHRH (G, 5 μM). Results are the mean ± SEM ($^{**}P < 0.01$ vs. c; $^{\#}P < 0.05$ vs. Aβ) (n=6). (**F**) Bax and (**G**) Bcl-2 assessed by Western blot on lysates from cells stimulated, for 24 h in control medium (c), with GHRH (G, 5 μM) or Aβ$_{1-42}$ (0.2 μM), either alone or in combination. Equal protein loading was determined by reprobing with antibodies to β-actin. Blots are representative of three independent experiments. Graphs represent the densitometric analysis of proteins normalized to β-actin and reported as percentage of control ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. c; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Aβ).

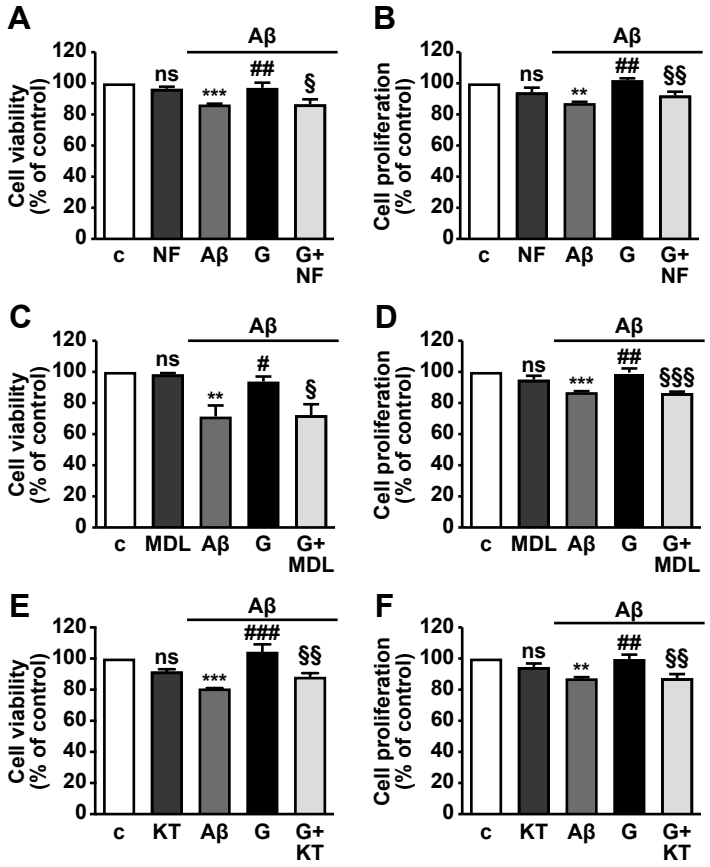

Figure 6. GHRH reduced $A\beta_{1-42}$-induced toxicity in NSCs through involvement of $G\alpha_s$/cAMP/PKA/CREB pathway. Cells were cultured in growth factor-deprived medium (c) for 2 h and then for further 24 h with $A\beta_{1-42}$, or NF449 (NF, 25 μM) (**A-B**), MDL12330A (MDL, 100 nM) (**C-D**), or KT5720 (KT, 100 nM) (**E-F**), either alone or in combination with GHRH (G, 5 μM). (**A-F**) Cell viability and proliferation were assessed by MTT assay and BrdU incorporation, respectively. The results are expressed as percentage of control and reported as means ± SEM ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. c; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Aβ; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ vs. Aβ + G; ns, not significant) (n = 4).

Phosphorylation of ERK1/2, PI3K/Akt and GSK-3β is essential for viability and proliferative effects of GHRH against $A\beta_{1-42}$-induced toxicity in NSCs

With regard to the signaling pathways and consistent with the previous results, GHRH which *per se* promoted both ERK1/2 and Akt phosphorylation, also counteracted $A\beta_{1-42}$-induced reduction of ERK1/2 and Akt phosphorylation and restored their activity even above the control levels, after 24 h of treatment. Moreover, the cells were incubated with the MEK inhibitor PD98059 (PD) or with the PI3K inhibitor wortmannin (W). Western blot analysis showed that both PD and wortmannin blocked GHRH-dependent ERK1/2 and Akt phosphorylation, in either absence or presence of $A\beta_{1-42}$ (Figure 7A and B).

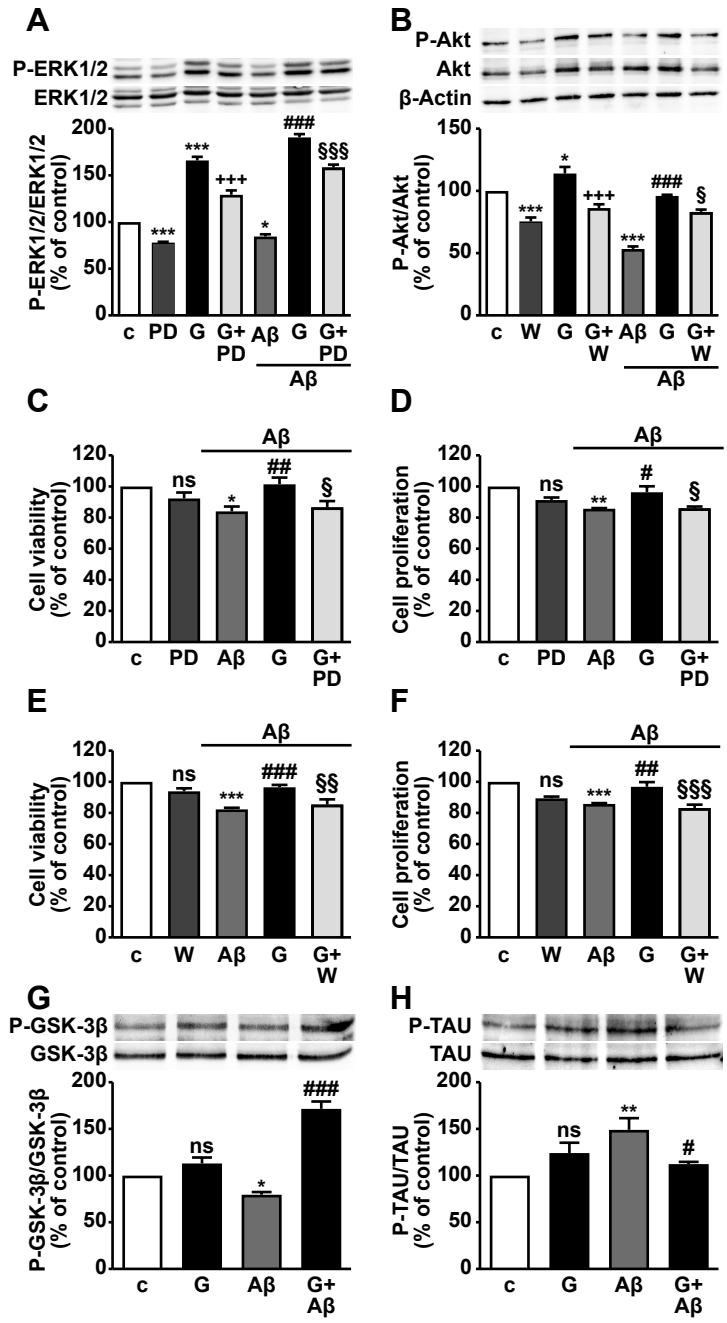

Figure 7. GHRH counteracts the detrimental effects of $A\beta_{1-42}$ through phosphorylation of ERK1/2 and PI3K/Akt, and inhibition of GSK-3β and Tau hyperphosphorylation. (**A**) ERK1/2 and (**B**) PI3K/Akt phosphorylation assessed by Western blot in cells treated with GHRH (G, 5 μM) or PD98059 (PD, 10 μM), or wortmannin (W, 25 nM), either alone or in combination with $A\beta_{1-42}$ (0.2 μM). For (**B**) β-Actin served as internal control. Blots were reprobed with total antibodies for normalization. Blots are representative of three independent experiments. Graphs show phosphorylated proteins normalized to total proteins and reported as percentage of control ($^{*}P < 0.05$, $^{***}P < 0.001$ vs. c; $^{+++}P < 0.001$ vs. G; $^{###}P < 0.001$ vs. Aβ; $^{§}P < 0.05$, $^{§§§}P < 0.001$ vs. Aβ + G; ns, not significant). (**C-F**) Cells were cultured in growth factor-deprived medium (c) for 2 h and then for further 24 h with $A\beta_{1-42}$, or PD98059 (PD, 10 μM) (**C-D**), or wortmannin (W, 25 nM) (**E-F**) either alone or in combination with GHRH (G, 5 μM). Cell viability and proliferation were assessed by MTT assay and BrdU incorporation, respectively. The results are expressed as percentage of control and reported as means ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. c; $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$ vs. Aβ; $^{§}P < 0.05$, $^{§§}P < 0.01$ $^{§§§}P < 0.001$ vs. Aβ + G; ns, not significant) (n = 4). Phosphorylation of GSK-3β (**G**) and TAU (**H**) assessed by Western blot on lysates from cells stimulated, for 24 h and 60 min respectively, in control medium (c), with GHRH (G, 5 μM) or $A\beta_{1-42}$ (0.2 μM), either alone or in combination. Blots were reprobed with total antibodies for normalization. Blots are representative of three independent experiments. Graphs show phosphorylated proteins normalized to total proteins and reported as percentage of basal ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. c; $^{#}P < 0.05$, $^{###}P < 0.001$ vs. Aβ; ns, not significant).

Furthermore, PD and wortmannin, which alone had no effect, blocked the protective action of GHRH against the detrimental effect of $A\beta_{1-42}$ also on cell viability and proliferation after 24 h of treatment (Figure 7C-F). Similarly, GHRH, which had no effects alone, blocked $A\beta_{1-42}$-induced inhibition of GSK-3β phosphorylation and restored GSK-3β activity even above the control levels, after 24 h of treatment (Figure 7G). GSK-3β is involved in the pathogenesis of AD and promotes hyperphosphorylation of Tau protein and formation of NFTs, which are among the classical hallmarks of AD [19,37]. Therefore, the effect of GHRH was examined on phosphorylation of Tau protein. GHRH alone had no effect, but abolished $A\beta_{1-42}$ -induced Tau phosphorylation, that was strongly increased with respect to untreated cells (Figure 7H). Overall, these data indicate that GHRH reduces $A\beta_{1-42}$ -induced toxicity in NSCs through phosphorylation of ERK1/2 and PI3K/Akt, inactivation of GSK-3β and inhibition of Tau hyperphosphorylation.

DISCUSSION

This study is the first to describe a novel neuroprotective role for GHRH in adult rat hippocampal neural stem cells (NSCs). Indeed, GHRH counteracted the detrimental effects of growth factor deprivation by promoting viability and proliferation and inhibiting apoptosis, through the involvement of GHRH-R signaling. Furthermore, GHRH exerted its mitogenic effects through mechanisms involving activation of the cAMP/PKA/CREB signaling and phosphorylation of ERK1/2 and PI3K/Akt, as well as inactivation of GSK-3β. In addition, the present study demonstrates that GHRH was able to induce a major differentiation of NSCs toward neurons and astrocytes. GHRH also reduced the negative effects of $A\beta_{1-42}$ on NSCs viability, proliferation, and apoptosis through ERK1/2 and PI3K/Akt pathway and inhibited both activation of GSK-3β and Tau hyperphosphorylation. Hippocampus is the principal brain structure associated with several cognitive functions such as emotional memory retention, learning mechanisms and affective behaviors, as well as neurogenesis [12,100]. Adult hippocampal neurogenesis comprises proliferation and differentiation of progenitors, survival and maturation of new neurons, and their integration into neuronal circuits [11,12]. Altered neurogenesis may reduce the plasticity of hippocampus, resulting in memory impairment and cognitive deficits, whereas enhanced neurogenesis represents a brain repair mechanism. Notably, impaired hippocampal neurogenesis has been shown to be a critical event in aging and disorders, including AD [101,102]. Different growth factors act as positive regulators of adult neurogenesis and improve cognitive function either directly, by promoting neurogenesis or indirectly, by exerting neurotrophic and survival effects in new neurons [14,103].

Despite of controversial data in literature about the role of the GHRH/GH/IGF-1 system in the development of senile dementia, it has been demonstrated that the GH/IGF-1 axis is

involved in brain growth and plasticity, proliferation and differentiation of NSCs [104,105], suggesting that as well GHRH could have a potential role in the regulation of cell survival. Thus, it seemed appropriate to investigate the possible role of GHRH on neurogenesis, which involves survival, proliferation, and differentiation of NSCs. GHRH was found here to potently stimulate cell viability and proliferation of NSCs and to prevent apoptosis induced by growth factor deprivation, suggesting neurogenic effects.

It is now well established that GHRH not only plays a central role in the release of GH from somatotroph cells, but growing evidence also demonstrated that this hormone is capable of exerting peripheral effects, being expressed in a variety of peripheral tissues, including lymphocytes, heart, skeletal muscle, placenta, ovary and testis, endometrial tissue, kidney, gastrointestinal tract, prostate, liver, lung, and peritoneal bone marrow-derived cells [42,52]. Furthermore, expression of GHRH and its receptor, GHRH-R, have been demonstrated also into cerebral cortex, cerebellum, and brain stem cells [50]. Here, the pituitary form of GHRH-R was found in NSCs, suggesting that GHRH exerted its effects by binding to its receptor. Involvement of GHRH-R was also bolstered by evidence that GHRH-R antagonist JV-1-36 abolished GHRH-induced viability and proliferation of NSCs.

GHRH proliferative and antiapoptotic actions have been previously reported by our and other groups in different cell types, such as fibroblasts, cardiomyocytes, and pancreatic islets, through the upregulation of cAMP/CREB signaling and ERK1/2, PI3/Akt and JAK2/STAT3 pathways. Particularly, GHRH has been shown to promote the proliferation and migration of mouse embryonic fibroblasts *in vitro*, and to accelerate healing in skin wounds of mice *in vivo* [63]. Moreover, different studies showed that both GHRH and its agonistic analogs promote cardioprotection; indeed, they increase survival of cardiomyocytes *in vitro*, improve cardiac function, protect against ischaemia–reperfusion injury in rat heart [47], reduce infarct size [57], attenuate cardiac hypertrophy [58] and induce cardioprotection after experimental myocardial infarction *in vivo* [69]. Furthermore, GHRH

agonists improves β-cells survival and reduce apoptosis in rat insulinoma cells [55] and stimulate insulin secretion from isolated rat islets *in vitro* [56].

GHRH-R is a $G\alpha_s$-protein coupled receptor which stimulates adenyl cyclase, converting ATP in cAMP, a second messenger involved in the GHRH signal transduction. cAMP/CREB signaling plays a central role in different steps of adult neurogenesis, including survival, proliferation, and integration of new neurons [21,97]. In NSCs, GHRH induced an early activation of cAMP and promoted CREB phosphorylation on Serine 133, the site required for the control of neurogenic transcriptional programs. In addition, the mitogenic actions of GHRH on cell viability and proliferation were blocked by adenylyl cyclase and PKA inhibitors, as well as by $G\alpha_s$ antagonists, suggesting GHRH-induced GHRH-R signaling through $G\alpha_s$/cAMP/PKA/CREB pathway. These results confirm previous data demonstrating an involvement of cAMP and CREB activation in the modulation of NSCs cell proliferation *in vivo* [21,97].

Accumulating evidence revealed that MAPK/ERK1/2 and PI3K/Akt pathways play a key role on the regulation of a variety of cell activities in hippocampal progenitors, including cell proliferation, migration and differentiation, and are involved in the mitogenic effects of different growth factors [17,106]. Interestingly, both ERK1/2 and PI3K/Akt pathways were found to promote CREB phosphorylation [18,23], suggesting a link between these pathways and neurogenesis of NSCs. Interestingly, our results show that GHRH induced the phosphorylation of ERK1/2 and Akt in NSCs, in agreement with previous findings in other cell types [47,48,57]. Moreover, inhibition of ERK1/2 and Akt by PD98058 and wortmannin respectively, reduced the GHRH-induced effects on cell viability and proliferation.

PI3K/Akt pathway exerts antiapoptotic effects via the phosphorylation of many effectors, including the cytoplasmatic protein GSK-3β. Inhibition of GSK-3β is crucial for the stabilization and nuclear translocation of β-catenin and activation of target genes [17,19]. GSK-3β/β-catenin signaling, a key component of the Wnt pathway, plays a crucial role in

progenitor cell differentiation and survival during neurogenesis [17,19]. Moreover, impaired GSK-3β activity has been associated with psychiatric disorders and neurodegenerative diseases, including AD [20,37]. In line with these data, GHRH rapidly increased the phosphorylation of the PI3K/Akt downstream target GSK-3β on Ser9, which resulted in its inactivation. Conversely, GHRH was unable to activate mTOR/p70S6K, the other downstream target of PI3K/Akt, involved in hippocampal synaptic plasticity, neurogenesis and autophagy [107,108]. Accordingly, Akt-induced phosphorylation and inhibition of GSK-3β were previously found to occur independently of mTOR, suggesting that Akt may independently activate one pathway or the other [48].

A limitation of this study is that the experiments were performed using a single cell type *in vitro*. However, the adult rat hippocampal neural stem cells (NSCs), employed herein, isolated from the hippocampus of adult Fisher 344 rats, are a well-established cell model, which have been thoroughly studied regarding their proliferative capacities and lineage stability [99]. Most importantly, are self-renewing cells with the capacity to differentiate into neurons, astrocytes, and oligodendrocytes, the three main lineages of the CNS [99]. The involvement of GHRH in cell differentiation has already been reported; for example, GHRH was found to induce differentiation and maturation of somatotroph cell in combination with acid retinoic and dexamethasone, although GHRH alone had no effect [38]. Moreover, GHRH promotes the differentiation of granulocytes from progenitor bone marrow cells into functional mature immune cells [60]. To our knowledge, this is the first evidence that GHRH is implicated in neural differentiation. However, it was already demonstrated that both GH and IGF-1 stimulate the genesis of neurons, astrocytes and oligodendrocytes [104] suggesting that also GHRH directly or indirectly, through the GH/IGF axis, may have a potential role on cell differentiation. This work confirms that NSCs could differentiate in neural lineages even in the absence of GHRH, as previously observed [99]. Furthermore, we observed that treatment with GHRH increased the mRNA levels of both *Tuj1* and *GFAP*, markers for

neuron and glia respectively. In addition, GHRH induced a significant increase of both NeuN and GFAP proteins, compared with untreated differentiated cells, suggesting that GHRH promotes the differentiation of NSCs towards neurons and astrocytes. However, further studies are needed to deepen this aspect. In fact, neuronal and glial cell replacement or supplementation could be beneficial for several neurological illnesses and thus constitute a novel therapeutic approach to neurological diseases such as AD.

Based on the protective effects observed in growth factor-deprived conditions and the signalling pathways involved, we next sought to determine whether GHRH would protect NSCs from the detrimental effects of $A\beta_{1-42}$ peptide. Indeed, Aβ is a main responsible for neuronal death, reduction of neurogenesis, memory, and cognitive impairment in AD [29,30]. Furthermore, AD, most common neurodegenerative disorder, is also characterized by accumulation of NFTs, abnormal phosphorylation of Tau protein, deposition of plaques in the brain and chronic inflammation [29,35]. Here, we show for the first time that GHRH counteracted the detrimental effects of $A\beta_{1-42}$ in NSCs, by increasing cell viability and proliferation and reducing apoptosis, through involvement of GHRH-R signaling and $G\alpha_s$/cAMP/PKA/CREB pathway. GHRH also increased both ERK1/2 and Akt phosphorylation, that was reduced by $A\beta_{1-42}$. In addition, ERK1/2 and Akt inhibition by PD98058 and wortmannin respectively, blocked GHRH-dependent ERK1/2 and Akt phosphorylation, in either absence or presence of $A\beta_{1-42}$.

Accumulating evidence suggest that Aβ modulates the proliferation and differentiation of NSCs via GSK-3β-mediated signaling [37]. Here, GHRH even promoted the phosphorylation/inactivation of GSK-3β, whose dephosphorylation/activation by $A\beta_{1-42}$. In the pathogenesis of AD, GSK-3β interacts with Tau, leading to its hyperphosphorylation, thus disrupting its normal function in regulating axonal transport and leading to the accumulation of NFTs and toxic species of soluble Tau [36]. Accordingly, this study shows

that GHRH also counteracted $A\beta_{1-42}$-induced phosphorylation of Tau and restored its phosphorylation to basal level in NSCs.

Overall, these findings are the first to show the novel protective role of GHRH in adult hippocampal NSCs exposed to different stress stimuli, through activation of survival and neurogenic pathways. In addition, the protection against the detrimental effects of Aβ and inhibition of signaling pathways characteristic of AD suggest a potential role for GHRH analogs as potential therapeutic compounds for reducing hippocampal cell loss, improving neurogenesis and for treating neurodegenerative disorders, such as AD, alone or possibly in combination with other drugs. Importantly, GHRH agonists are able to cross the blood-brain barrier, a fundamental requisite for their therapeutic use at the central level [109]. Thus, future *in vivo* studies are needed to clarify the mechanisms of action of GHRH analogs on hippocampal neurogenesis and neuroprotection, possibly leading to their development as pharmacological tools for AD and other neurodegenerative disorders.

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