Ghrelin and olfactory cells:

effects on food-intake modulation, as trophic support and its possible role in differentiation.

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Doctoral Dissertation

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SUMMARY

Ghrelin (Ghre), a gut-brain peptide hormone, and its receptor (GHS-R 1a) are expressed in several parts of the organisms, such as olfactory bulbs (OB). In the central nervous system, Ghre has been mainly observed in hypothalamic neurons, but it has also been reported its synthesis in the OB, amygdala, pyramidal neurons of the cerebral cortex and the dorsal vagal complex of the medulla oblongata. Moreover, it is known that Ghre is involved in cognitive mechanisms and eating behaviour, in fact, its expression increases in anticipation of food intake. In the olfactory system, during development, peculiar glial cells, known as Olfactory Ensheathing Cells (OECs), have the duty to drive the non-myelinated axons of the Olfactory Receptor Neurons from the olfactory mucosa to OB. The OECs show stem cell characteristic and they secrete different neurotrophic factors, promote axonal growth, and they also act as mechanical support. OEC transplantation has emerged as a possible experimental therapy to induce repair of nervous system injury, even if the functional recovery is still limited.

Our neuroanatomic study provides evidence on support for the Ghre modulation of smell. These results show that Ghre neuron projections from the mitral cells of the OB can send olfactory information via branching connections to the amygdala and the hypothalamus. This pathway could play an important role in regulating feeding behavior in response to odours.

The presence of Ghre in the OB prompted us to verify and investigate on the secretion and the presence of Ghre peptide and its receptors in

OECs. Our results, for the first time, showed that OECs were able to secrete Ghre and to present its GHS-R 1a.

Following this, we wanted to verify the effect of the Ghre on viability and on the expression of some neural markers, such as Nestin, Glial Fibrillary Acid Protein, Neuregulin, and β-III-tubulin, in the OEC cultures. Our results demonstrate that Ghre was able to stimulate the OEC viability and to modify the expression of some of these biomarkers. In particular, it was observed a loss of stem cell feature and therefore the possible orientation towards an adult neural phenotype.

Therefore, the data gathered in this thesis suggest the important role of Ghre as a protective neuropeptide.

It can be hypothesized that Ghre and GHS-R 1a can interact as enhancement function, in the peripheral olfactory circuit, providing neurotrophic support to the synaptic interaction between the ORNs and the mitral cells. Moreover, since it is known that Ghre has a neurotrophic action, both in promoting axonal growth and synaptogenesis probably through the secreting factors, it could be hypothesized that even the OECs through the Ghre, can improve the growth of neurites and promote alignment and it is possible to consider this peptide as potential support for the OECs in the transplantation therapy.

INTRODUCTION

Ghrelin (Ghre) is mostly recognized as a small orexigenic peptide gut hormone produced in the stomach, but it exists also in several part of the brain, such as hypothalamus, amygdala, hippocampus and olfactory system (Kojima et al. 1999; Lu et al. 2002; Carlini et al. 2004; Ferrini et al. 2009; Russo et al., 2017). It plays an important role in appetite stimulation, feeding behavior, energy homeostasis, and carbohydrate metabolism (Cowley et al. 2003; Muller et al. 2015).

Recently, it has attracted particular interest for its neuroprotective, antioxidant, anti-inflammatory and anti-apoptotic properties (Can et al., 2015; Popelová et al. 2018; Fallahi et al., 2019).

The human Ghre gene is located on chromosome 3 (3p25-26) and encodes a pre-pro-hormone of 117 amino acids. Ghre becomes a bioactive peptide following the post-translational addition of n-octanoic and n-decanoic acids by the action of the enzyme Ghre-O-acyltransferase (GOAT), a critical modification to allow the link with its endogenous ligand for the GH secretagogue receptor (GHS-R1a) (Kojima et al., 1999; Date et al., 2000; Van der Lely et al., 2004; Yang et al., 2008; Zaniolo et al., 2011). This receptor was widely found in both central and peripheral tissues (Guan et al., 1997), including hypothalamus, hippocampus, substantia nigra, and olfactory bulb (OB), and in other regions (Mani et al., 2014; Rhea et al., 2018).

This ubiquity underlines the working spectrum of Ghre.

Ghre the “hunger hormone”.

A large literature shows that there is a close communication between gut and brain, a reciprocal axis capable of intervening on emotional states, decision-making process through the attendance of components such as peptides, neuropeptides, lipopolysaccharides and peptidoglycanides (Holzer et al., 2014; Carabotti et al., 2015; Arneth et al., 2018; Lach et al., 2018). Ghre is commonly known as the "hunger hormone", numerous studies have shown that in the pre-prandial stage, the levels of Ghre increase (Cummings et al., 2001). It is released by the gastric cells (X/A-Like) of the stomach and is able to cross the Blood Brain Barrier (BBB) connecting the central and peripheral systems (Banks, 2008; Bayliss et al., 2016; Howick et al., 2017).

Ghre's orexigenic activity is at the base of feeding processes. In particular, Ghre plays a role in the olfactory processing necessary for the search for food in rodents as it improves exploratory sniffing (Tong et al., 2011). Many studies clearly show that Ghre and its receptors are expressed in the OB and other brain centers, such as the hypothalamus, amygdala and hippocampus (Zigman et al. 2006; Tóth et al. 2010; Alvarez-Crespo et al. 2012; Russo et al. 2017).

The sense of smell informs about the presence of palatable food through both by a local circuit, inside the OB, and by a centrifugal system that processes and modulates this information outside (Saper et al. 2002; Trellakis et al. 2011). Previous studies highlighted the existence of collateralized Ghre neurons, directed from the hippocampus to the amygdala and hypothalamus (Russo et al. 2017).

Ghre the trophic support and its possible role in differentiation.

Recent papers on Ghre neuropeptide describe it for its peculiar functions as a neuroprotective factor of neurodegenerative diseases in various animal models (Moon et al. 2011; Can et al. 2015), as an anti-inflammatory and anti-apoptotic factor; in addition, it is also able to reduce the oxidative stress (Lee et al., 2016; Huang et al., 2019; Qu et al. 2019). It plays a role in the regulation of neuroendocrine and neurodegenerative processes, especially in learning and memory consolidation (Panagopoulos et al., 2014; Jiao et al. 2017).

Several studies show that Ghre has a part also in synaptic formation and neurogenesis in both in vivo and in vitro model. It supports the synaptic formation improving their recovery and the neurite growth (Stoyanova et al. 2016; Liu et al. 2019), stimulates adult hippocampal neurogenesis for learning and memory. Moreover, Ghre acts promoting the regeneration of the hippocampal dendritic spines (Berrout et al., 2012; Kent et al. 2015).

Recently, it was demonstrated that Ghre plays a role on synaptic formation and neurogenesis. Some authors highlighted that Ghre stimulates synaptic formation in cortical cultures improving synapse recovery (Stoyanova et al. 2016); in vivo and stimulates adult hippocampal neurogenesis for learning and memory (Kent et al. 2015), reorganizing the dendritic spines of the hippocampal neurons and promoting their regeneration, through the continuous activation of its receptor (Berrout et al., 2012).

Olfactory Cells

The mammalian olfactory system has the peculiarity to support neurogenesis throughout their constant lifetime (Graziadei et al., 1979; Pellitteri et al., 2010). The ability of the Olfactory Receptor Neuron (ORNs) to stimulate neurogenesis and to realize new synapses takes place under the control of special glial cells known as Olfactory Ensheathing Cells (OECs) (Graziadei et al., 1980; Doucette et al., 1990). Their role is to unsheath the olfactory nerve fibers, migrate and drive the non-myelinated axons of the ORNs from the olfactory mucosa towards the OB (Ramon-Cueto et al., 1998; Fairless et al., 2005; Windus et al., 2011; Ekberg et al., 2012; Nazareth et al., 2015).

The OECs provide mechanical support to the olfactory axons (Barton et al., 2017). They share similarities with Schwann cells, in fact, they express some characteristic markers such as adhesion molecules, such as laminin, fibronectin, trophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophic factor derived from glial cells (GDNF) (Woodhall et al., 2001; Moreno-Flores et al., 2002).

Several therapy studies for spinal cord injuries showed that OECs transplantation, have the ability to promote axon regeneration and provide neuroprotection of injured axons into the damaged spinal cord (Lu et al., 2001; Lu et al., 2002; Yang et al., 2015). Therefore, the OEC, through the Ghre, could intervene as a reinforcing factor, in the peripheral olfactory circuit, providing neurotrophic support to the

synaptic interaction between the ORNs and the mitral cells, improving the growth of neurites and promoting their alignment.

AIMS OF THIS THESIS

The general objective of this thesis was to investigate the Ghrelin effect on the olfactory cells.

More specifically, the main aims were:

I. Ghrelin release from the mitral cells can activate a collateralized direct pathway among olfactory bulb, amygdala and hypothalamus in response to the perception of an appetizing smell.

II. Identify the presence of the Ghrelin peptide and its GHS-R 1a receptor in OECs.

III. Study the effect of Ghrelin on OECs to visualize potential orientation towards a neuronal phenotype.

IV. Asses an *in vitro* model to study the OECs neuroregenerative capacity, after neural injury.

RESULTS

Ghrelin-containing neurons in the olfactory bulb send collateralized projections into medial amygdaloid and arcuate hypothalamic nuclei: neuroanatomical study.

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Abstract

Ghrelin, a gastrointestinal hormone, is a modulator of the sense of smell. The main source of ghrelin in the central nervous system has been mainly observed in specific populations of hypothalamic neurons. An increasing number of studies have reported ghrelin synthesis and its effect on neurons outside the hypothalamus. Ghrelin and its receptors are expressed in the olfactory bulbs and in other centres of the brain, such as the amygdala, for processing olfactory signals, pyramidal neurons of the cerebral cortex and the dorsal vagal complex of the medulla oblongata. It is known that ghrelin is involved in cognitive mechanisms and eating behaviours, in fact, its expression increases in anticipation of food intake. In order to identify the existence of centrifugal direct afferents from the main olfactory bulb to the medial amygdala and the hypothalamus arcuate nucleus, in this work we used

two retrograde tracers, Dil and Fluoro Gold, and immunohistochemical procedure to visualize
positive ghrelin neurons. Our paper provides neuroanatomic support for the ghrelin modulation of smell. Our results show that ghrelin neuron projections from mitral cells of bulbs can transmit olfactory information via branching connections to the amygdala and the hypothalamus. This pathway could play an important role in regulating feeding behaviour in response to odours.

Keyword

Ghrelin · Olfactory bulbs · Retrograde tracers · Immunohistochemistry · Feeding behaviour · Rat

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Introduction

Ghrelin in the central nervous system

Ghrelin (Ghre), an orexigenic peptide, plays a critical role in the control of feeding behaviour (Carlini et al. 2004). It is predominantly produced in the stomach but low levels of Ghre are also expressed in the brain (Lu et al. 2002); the main sites of Ghre synthesis are in paraventricular, dorsomedial, arcuate, ventromedial and lateral hypothalamic nuclei (Kojima et al. 1999; Ferrini et al. 2009).

By using RIA, HPLC and immunolabelling techniques, Ghre was identified in hypothalamic sites, but Ghre-immunopositive staining was also detected in the pyramidal neurons of the cerebral cortex and the dorsal vagal complex of the medulla oblongata (Cowley et al. 2003; Sato et al. 2005; Hou et al. 2006). Ghre projections are described outside the hypothalamus including the amygdala (Burdyga et al. 2003). These findings hypothesize an involvement of Ghre in neural transmission and neuronal function (Lu et al. 2002). Therefore, Ghre has a strong physiological significance and biological function on homeostasis, metabolism and thus feeding behaviour as has been authoritatively reviewed (Cowley and Grove 2004; Jobst et al. 2004; Olszewski et al. 2008).

Ghrelin and sense of smell

The sense of smell informs about the presence of palatable food and therefore determines the hedonic value of it, thus influencing food

intake (Saper et al. 2002; Trellakis et al. 2011). The olfactory information is not only mediated by an internal local loop of the olfactory bulbs (OB), but also by a centrifugal system that processes and modulates.

The functional link between the OB and other olfactory areas of the brain is well known; in particular with the hypothalamus, amygdala and hippocampus (Kang et al. 2009; Gourévitch et al. 2010; Gascuel et al. 2012). At the level of the olfactory epithelium, circulating hormones such as leptin, adiponectin, orexins and Ghre modulate the olfactory sensitivity and the olfactory response (Hass et al. 2008; Savigner et al. 2009; Sun et al. 2016). In particular, Ghre is assumed to be a modulator of the sense of smell and thus of body weight and the power state. Many studies provide clear evidence that Ghre and its receptors (GHS-R) are expressed in the OB (both main—MOB and accessory—AOB areas) and in other centres of the brain, such as the hypothalamus, amygdala and hippocampus for processing olfactory signals (Merzhanova et al. 2000; Zigman et al. 2006; Tóth et al. 2010; Alvarez-Crespo et al. 2012; Sun et al. 2016; Russo et al. 2017). This suggests that Ghre is involved in the physiology of the olfactory processes that modulate the activity of the olfactory circuit triggered by smells, as well as being involved in voluntary control of sniffing in other brain regions, including the sensory motor cortex and brainstem (Tong et al. 2011).

Ghrelin, olfactory circuit and feeding behaviour

Ghre and GHS-R directly modulate the odour-induced activity in central olfactory circuits. GHS-R were identified in OB, both at the level of glomerular and mitral layers of the MOB and at the AOB, suggesting that Ghre is involved in olfactory processing through the activation of the MOB. Therefore, Ghre can affect the olfactory function by modulating, along the olfactory circuit, the OB responses to odours. Thus, it is reasonable to think that the OB express neuropeptide Ghre as well as the GHS-R. The medial amygdaloid nucleus of the amygdala (Me) is one of the main structures receiving direct projections from the MOB (Cowley et al. 2003; Kang et al. 2011) and is a critical node to integrate olfactory information. Several anterograde tracing studies showed that many olfactory structures give rise to projections into the Me (Pitkänen et al. 2000; Cádiz-Moretti et al. 2016). In fact, a direct connectivity between the MOB and the medial amygdala (Me) was discovered, receiving input primarily from a different chemosensor (Bader et al. 2012). Recent studies have shown that there is a subpopulation of MOB mitral cells that project directly into the Me (Pro-Sistiaga et al. 2007; Kang et al. 2009). Moreover, intra-amygdala Ghre injections demonstrate the presence of GHS-R in the amygdala (Alvarez-Crespo et al. 2012). These data suggest that Ghre's neurobiological effects in this brain area are linked to food intake (Egecioglu et al. 2011; Skibicka and Dickson 2011).

The amygdala, responsive to Ghre, is therefore important in appetitive learning, for the emotional and motivational meaning that attributes to

the feeding stimuli (Burns et al. 1994; Baldwin et al. 2000). It is possible that Ghre may reach other areas of the brain related to the sense of smell and olfactory memory, such as the hypothalamus and hippocampus. Our previous studies highlighted the existence of collateralized Ghre-neurons, directed from the hippocampus to amygdala and to hypothalamus (Russo et al. 2017). In addition, it is conceivable that the relationship between the amygdala and OB can justify the link between the effects of Ghre on food intake and emotional capacity to mediate the activities related to foraging for food (Alvarez-Crespo et al. 2012). In fact, the OB, playing an important role in food intake, contribute to estimate the hedonic properties of food (Caba et al. 2014). It is necessary for a normal pattern of feeding behaviour. There are limited responses that suggest direct connections between OB and the hypothalamus: Gascuel et al. (2012) described projections from the olfactory bulbs to the hypothalamus of the POA regions, while Price et al. (1991) highlighted positive data on the electrical stimulation of the olfactory bulb consistently found in the postero-lateral hypothalamus, but only occasionally at more rostral levels.

Many studies of interneuronal interactions among different brain areas have shown a close correlation between olfactory amygdala and the hypothalamus. The Ghre axis provides the perfect signalling system for feeding and responds to emotional arousal and stress (Labarthe et al. 2014; Müller et al. 2015). Ghre induces an orexigenic effect through neuropeptide Y (NPY)-containing neurons in the arcuate nucleus

(ARH) that are likely the primary target playing the most important role in feeding behaviour. The co-expression of GHS-R and NPY mRNA in ARH neurons suggests a direct action of Ghre on NPY cells in the ARH (Wang et al. 2002). The amygdalo-hypothalamic interactions form a part of a complex morpho-functional system that is involved in the functional emotions of feeding behaviour. In addition, there is now evidence in the rat (Pro-Sistiaga et al. 2007) for direct connections from MOB mitral cells to the Me, suggesting that odours processed by the main olfactory system, via the Me, may have greater access to the hypothalamus than previously realized. The projection roadmap from the MOB to the Me increases the possibility that a direct pathway from the MOB to amygdalo-hypothalamic structures may exist in mammals, including humans (Kang et al. 2009). The presence of GHS-R in the olfactory circuits (MOB-Me-ARH), shows a new olfactory modulation mechanism, through which energy state of an organism can interact with the ability to process the olfactory chemosensors (Egecioglu et al. 2011). In order to identify the existence of centrifugal afferents from the MOB to Me and ARH, we used two fluorescent retrograde tracers, Dil and Fluoro Gold, and the immunohistochemical detection to visualize positive Ghre-neurons. The target of our study was to confirm the hypothesis that the presence of Ghre within MOB mitral (M) cells can activate a collateralized direct pathway among OB, Me and ARH, in response to the perception of an appetizing smell.

Materials and methods

Animals

Nine adult male Sprague-Dawley rats (provided by Envigo RMS s.r.l. Italy) housed under 12 h light/dark conditions with ad libitum access to food and water, were used in this study. Animals were allowed to acclimatize for at least 1 week before being used in the experiments. All the experimental

procedures were carried out according to the Italian Guidelines for Animal Care (D.L. 116/92 and 26/2014), which are in compliance with the European Communities Council Directives (2010/63/EU) and were approved by the Ethical Committee at the University of Catania. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental procedures

The anaesthetized rats were placed in a Kopf stereotaxic frame and monolaterally injected with the following tracers: 0.04 μl of freshly dissolved Fluoro Gold (FG, Biotium; diluted 6% in saline solution;) into the ARH nucleus, at the following coordinates (anterior-posterior AP = − 3.30 mm; lateral L = 0.20 mm; vertical V = − 10.20 mm); freshly dissolved 0.04 μl Dil (1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes, Inc., solubilized in 5% N,N-dimethylformamide) successfully used it in previous studies (Mameli et al. 2009, 2016) into the Me, on the same side, at the following coordinates (AP = − 3.30 mm; L = 3.00 mm; V =

− 9.40 mm) (Paxinos and Watson 1998) as shown in Fig. 1A, B. Both tracers were pressure-injected at a rate of 50 nl/min using 1 μl Hamilton microsyringe advanced by an electronic Microdrive (David Kopf). These tracers were chosen because they are used to direct label nerve fibres (Russo et al. 2017).

Seven days after the injections, the animals were anaesthetized again and perfused through the ascending aorta with 60 ml saline, followed by 300 ml ice-cold 4% paraformaldehyde phosphate buffer (pH 7.4). The bulbs and the brains were removed, immersed in the same fixative for 3–4 h and then cryoprotected overnight in phosphate-buffered 20% sucrose solution.

Coronal sections (10 μm) of bulbs and brains (30 μm) were cut by using a cryotome and collected in phosphate buffer (PBS, pH 7.4) and serially mounted on slides.

The injection zones were identified and observed under a Nikon Eclipse 80i fluorescent microscope carefully set and images were captured with a digital camera (Nikon). Then only the animals (seven) in which the tracer was strictly confined within the ARH and Me nuclei were considered for the results, while the others (two) were used as negative controls. The distribution of the tracers in the main olfactory bulb neurons was identified by using selective filters for Dil (rhodamine, 560-nm wavelength) and FG (wide band ultraviolet-UV excitation filter, 360–390 nm wavelength), in order to highlight the single and/or doublelabelled neurons. We observed the same neuron labelled with two or three markers with different filters, without moving the field.

The fluorescent retrogradely labelled neurons were highlighted into the MOB. The fluorescent microscopic detection of MOB neurons was taken at low (4×, Fig. 1F; 10×, Fig. 1C, D) and high (40×, Fig. 1G–I, L) magnifications from serial sections. High magnification was used to estimate the population of double-labelled MOB neurons.

Immunohistochemical procedures

Only the area corresponding to the double-labelled neurons in MOB was processed immunohistochemically for the detection of Ghre. After washing in PBS, bulbar coronal sections were treated with PBS containing 5% normal goat serum (NGS), 0.1% Triton X-100 at room temperature

(RT) for 15 min, and then with rabbit anti-ghrelin (Santa Cruz Biotechnology Inc., Dilution 1:200) overnight at 4 °C. After rinsing in phosphate-buffered saline (PBS), the sections were incubated for 30 min in a secondary antibody, goat anti-rabbit antibody IgG Fluorescein Isothiocyanate (FITC, 1:200; Merck KGaA), diluted in PBS plus Triton X-100 and NGS (1%). Sections were air-dried and observed using a Nikon Eclipse 80i fluorescence microscope equipped with filter combinations revealing red (Dil), yellow (FG) and green (FITC) fluorescence. The selective filter (FITC, 450–520 nm wavelength) was used to assess the distribution of the Ghre-mono-labelled and triple-labelled MOB neurons. Images were captured with a digital camera (Nikon).

Data analysis

To determine the number of bulbar single, double and triple-labelled neurons, the cell count was performed on each individual coronal sections. The incidence of triple-labelled cells was estimated directly from the immunohistochemically series processed by sequentially viewing tissues with the three different filters. For every animal, three sections were evaluated and the labelled cells counted. Thus, cell number was expressed as the average number/section calculated from these three sections. The occurrence of one or more fluorochromes within the same cell body indicates both the direct and collateralized projection model and its chemical nature.

Results

Injection sites

In order to label afferent connections directing to the medial amygdaloid nucleus and to the arcuate hypothalamic nucleus originating from olfactory bulbs. The most representative of right injection site is shown in Fig. 1; injections were confined at Me (Fig. 1C) and ARH (Fig. 1D), respectively. The postero-medial cortical amygdala as well as the ventromedial hypothalamic nucleus were completely not included in the injection site.

Responses to the retrograde-labelling

In this study, we show that injection of FG into the ARH and Dil into the Me resulted in variable numbers of retrogradely labelled neurons in the MOB (Fig. 2; Table 1). The resulting retrograde-labelling was analysed in the different layers of the olfactory bulb, particularly in the granule cell (GrL) and glomerular (GL) layers as well as in the caudoventral DG, specifically in the mitral cell layer (MCL).

The retrograde-labelled neurons involved in these projections were only identified in the MCL of the ipsilateral OB, compared to the injection sites, while they were absent in the other layers of OB. These labelled mitral cells belonging to the MOB are localized between internal and external plexiform layers of bulb and extend their corresponding axons directly to Me and ARH (Fig. 1E, F).

Within the MOB, the mitral cells (MC) were analysed in the MCL at the fluorescent microscope. The mitral cells were easily identified as they are bigger and with triangle shape compared with tufted cells. No tufted cells within the layers were highlighted. An average of 40 ± 1.0 retrogradely labelled mitral cells (lMC) distributed at levels from 6.70 to 6.20 mm from bregma was detected (Fig. 2; Table 1). The highest concentrations of lMC were seen in the 6.70-mm plane from the bregma (Fig. 1E, F).

Distribution of the retrograde-labelling and Ghre-positive neurons in the MCL of the MOB

FG-mitral-single- labelled cells (FG-slMC), hypothalamic ARH-projecting neurons (Fig. 1E; Table 1) were rather sparsely distributed

in the MCL (5 ± 0.1 FG-slMC), while Dil-mitral-single-labelled cells (Dil-slMC), amygdaloid Meprojecting neurons (Fig. 1E; Table 1) were uniformly located in the medial portion of the MCL (12 ± 0.4 Dil-slMC). The double-labelled retrogradely mitral cells (18 ± 0.3 dlMC) were highlighted in greater number in the medial portion of the MCL, while the distribution decreased in the lateral portion. A relatively low number of FG/Dil/FITC triple-labelled mitral cells (5 ± 0.2 tlMC; ranged in size from 23 to 30 μm) exhibiting Ghre-like immunoreactivity with FITC (Fig. 1E, G; Table 1) was scattered mainly in the medial portion of the MCL (about 3–4% of the total Ghre-immunoreactive population). Ghrelin staining was only somatic, it was not visible also in the proximal dendritic arbour. Single and double-labelled cells (size from 10 to 20 μm) were found in the MOB as shown in Fig. 1H, I, L.

Discussion

It is universally recognized that Ghre is present in the hypothalamic nuclei and that in this district it represents the most important endogenous source of the hormone in the CNS (Kojima et al. 1999; Sato et al. 2005; Yoon et al. 2005). However, the existence of extra-hypothalamic Ghre-synthesizing neurons is not yet accepted by all researchers (Cabral et al. 2017). Recently the function of Ghre peptide in the CNS has found remarkable interest, particularly as regards its role in olfactory processes. Many authors have reported that responses to odours that regulate eating behaviours are mediated by anatomic pathways involving the hypothalamus and the amygdala (Saper et al.

2002; Cowley et al. 2003; Shepherd 2006; Kang et al. 2011; Trellakis et al. 2011). There is evidence for direct connections from MOB-MC to Me (Pro-Sistiaga et al. 2007), suggesting that odours are transmitted through MOB to Me. There is still no evidence of direct connections from MOBMC to the arcuate core of the hypothalamus, though smells processed by MOB through Me may have preferential access to the hypothalamus. The afferent Ghre-signal to the amygdala could be blood-borne (Alvarez-Crespo et al. 2012), but it could equally be centrally derived. It is known, Ghre influences olfactory function in rodents and humans (Tong et al. 2011).

Our work provides neuroanatomic support for the Ghre modulation of smell, suggesting a Ghre pathway modulating the olfactory responses at hypothalamic and amygdaloid levels. In fact, these areas are a key brain target for Ghre, integrating effects of food intake and emotional reactivity. Therefore, our results support a major influence of Ghre on food intake, although it is clear that the effects produced by Ghre are not limited to this, but tend to put the body into the condition of recovering its ideal energy status (Ferrini et al. 2009).

It is firmly established that the responses to odours regulating feeding behaviours are mediated by pathways that involve the hypothalamus and amygdala (Cowley et al. 2003; Kang et al. 2011; Trellakis et al. 2011). The direct projections that we have shown suggest that Ghre axonal fibres from MOB-MC can transmit olfactory information via branching connections to the amygdala and hypothalamus, in agreement with some authors (Kang et al. 2009). We found that MOB

mitral cells extend collaterals to the amygdaloid Me and hypothalamic ARH targets. This pilot study highlights the presence of Ghre at the mitral cell level of the MBO: in fact, a good number of Ghre positive lMC supports the hypothesis that Ghre could modulate the olfactory response to smell.

In conclusion, this explorative neuroanatomical study aimed to test the hypothesis that the central effects of the peptide, through these pathways might regulate feeding behaviour responding to odours (Fig. 3).

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Conflicts of interest

The authors declare that they have no conflict of interest

Ethical Approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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Figure 1

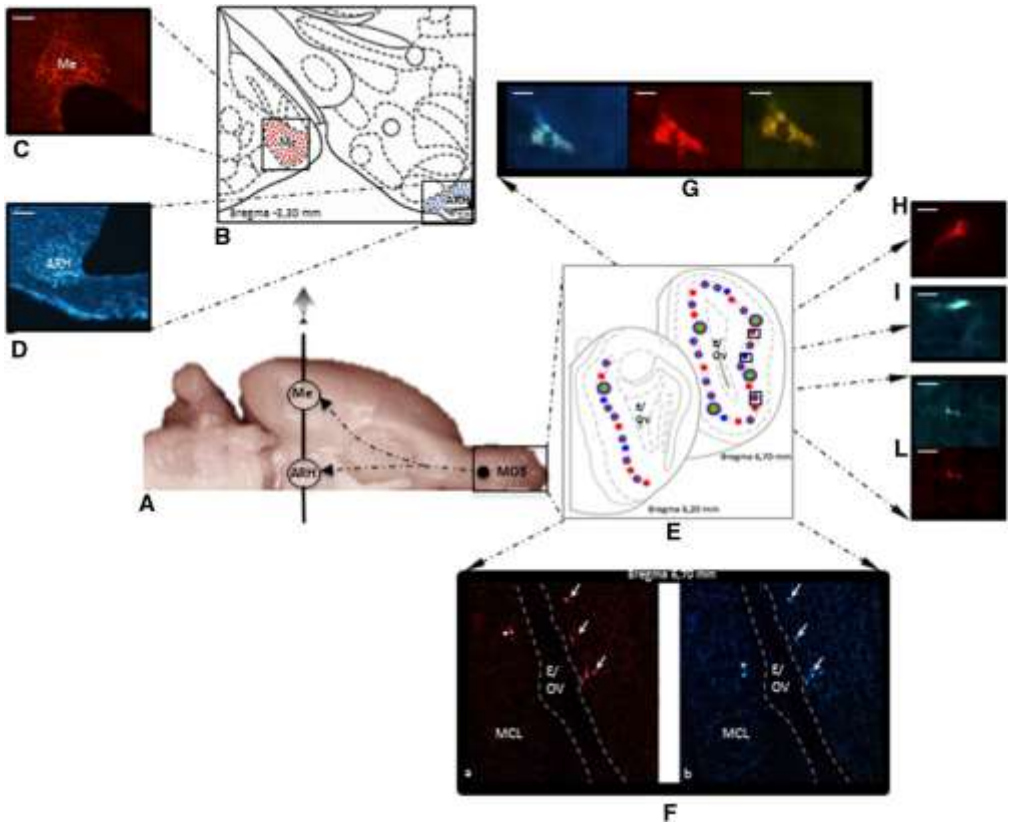

Fig. 1. A Schematic representation of Fluorogold (FG)/Dil/FITC triple-labelled neurons (tlMC), positive Ghre-neurons are labelled with FITC, which directly projects to Me and ARH, superimposed onto a photomicrograph of a rat brain (dashed black line). **B** Schematic drawing that shows the brain sites of Dil and FG injections (modified from Paxinos and Watson 1998); **C, D** Representative injection sites: fluorescent micrograph of examples of Dil injected area (Me); fluorescent micrograph of examples of FG injected area (ARH). Scale bar, 200 μm. **E** Coronal diagram of ipsilateral projection from the olfactory bulb to the Me and ARH. Symbols indicate the location of labelled cells in the mitral cell layer (MCL): blue dot = single-FG-labelled neurons (FG-slMC); red dot = single-Dil-labelled neurons (Dil-slMC); blue and red concentric circle = double-FG/Dillabelling neurons (dlMC); blue, red and green concentric circle = FG/ Dil/FITC triple-labelled neurons (tlMC). Each symbol is equivalent to one labelled neuron. **F**: Fluorescent micrograph (magnification ×4) indicates the (a) Dil and (b) FG labelled layer (MCL) of the MOB: arrows = dlMC; rhomb = Dil-slMC; asterisk = FG-slMC. **G**: Example of FG/Dil/FITC triple-labelled neuron in the MOB (size 25 μm). **H** Example of single-Dil-labelled neurons in the MOB (size 20 μm). **I** Example of single-FG-labelled neurons in the MOB (size 20 μm). **L** Example of double-FG/Dil-labelling neurons in the MOB (size 10 μm). Scale bar 10 μm

Figure 2

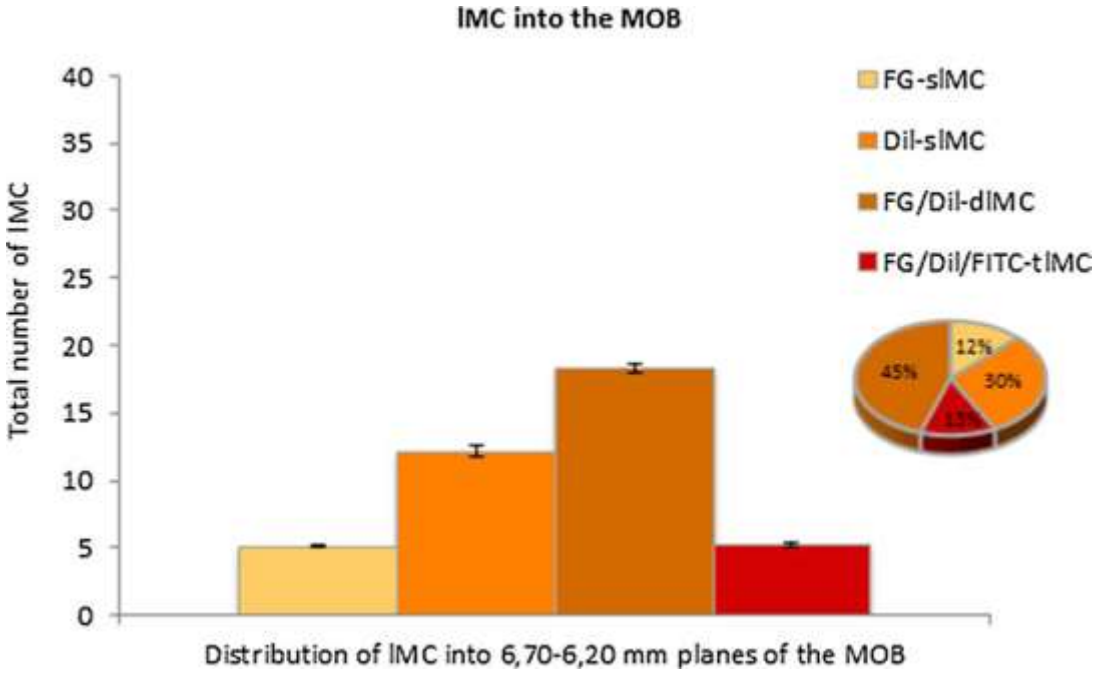

Fig. 2 The graph shows the highest localization of total labelled mitral cell (lMC) in the MOB at 6.70–6.20 mm planes from the bregma according to Paxinos and Watson (1998). Pie chart indicates the percentage localization of single, double and triple-labelled neurons at 6.70–6.20 mm planes of the MOB

Table 1

Olfactory bulb MOB planes	(a) FG	(b) DiI	(c) FG/DiI	(d) FG/DiI/FITC
6.70 mm	3 ± 0.07	8 ± 0.26	12 ± 0.18	4 ± 0.15
6.20 mm	2 ± 0.03	4 ± 0.14	6 ± 0.12	1 ± 0.05
Total lMC	5 ± 0.1	12 ± 0.14	18 ± 0.3	5 ± 0.2

Table 1 Localization of the retrograde-labelling and Ghre-positive neurons (lMC) into the mitral cell layer (MCL) of MOB (6.70 and 6.20 mm planes from bregma according to Paxinos and Watson 1998)

Figure 3

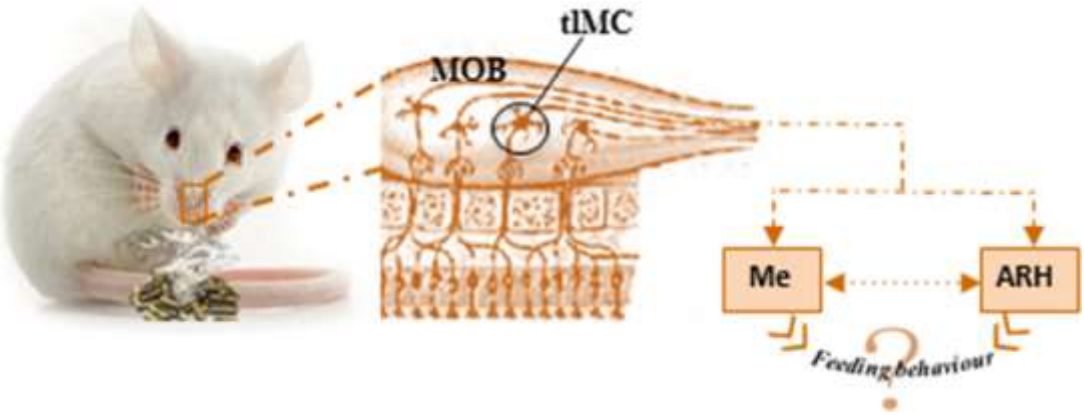

Fig. 3 The figure shows a schematic view of our proposed mechanism, for which Ghre might promote food intake (orange arrows) responding to odours. Circled triple-labelled mitral cell (tlMC) project Ghre signal to the amygdala (Me) and hypothalamus (ARH) (orange dashed line), connected between them (orange dash-dot arrow)

Olfactory Ensheathing Cells express both Ghrelin and Ghrelin Receptor in vitro: a new hypothesis in favor of a neurotrophic effect.

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Abstract

Olfactory Ensheathing Cells (OECs) are glial cells able to secrete different neurotrophic growth factors and thus promote axonal growth, also acting as a mechanical support. In the olfactory system, during development, they drive the non-myelinated axons of the Olfactory Receptor Neurons (ORNs) towards the Olfactory Bulb (OB). Ghrelin (Ghre), a gut-brain peptide hormone, and its receptor (GHS-R 1a) are expressed in different parts of the central nervous system. In the last few years, this peptide has stimulated particular interest as results show it to be a neuroprotective factor with antioxidant, anti-inflammatory and anti-apoptotic properties. Our previous studies showed that OB mitral

cells express Ghre, thus being able to play an important role in regulating food behavior in response to odors. In this study, we investigated the presence of Ghre and GHS-R 1a in primary mouse OECs. The expression of both Ghre and its receptor was assessed by an immunocytochemical technique, Western Blot and Polymerase Chain Reaction (PCR) analysis. Our results demonstrated that OECs are able to express both Ghre and GHS-R 1a and that these proteins are detectable after extensive passages in vitro; in addition, PCR analysis further confirmed these data. Therefore, we can hypothesize that Ghre and GHS-R 1a interact with a reinforcement function, in the peripheral olfactory circuit, providing a neurotrophic support to the synaptic interaction between ORNs and mitral cells.

Keyword

Olfactory Ensheathing Cell cultures, Ghrelin, Ghrelin receptor, Western blot, Immunocytochemistry, Polymerase chain reaction, Neurotrophic effect

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1. Introduction

1.1. Olfactory Ensheathing Cells in the olfactory system

A characteristic of the mammalian olfactory system is the constant lifetime neurogenesis (Pellitteri, Spatuzza, Stanzani, and Zaccheo, 2010). After Olfactory Receptor Neuron (ORN) death, new neurons are produced starting from the mitotic division of the basal cells belonging to the olfactory epithelium layer (Graziadei and Monte-Graziadei, 1979; Mackay-Sim and Kittel, 1991; Doucette, 1990; Pixley, 1992). The capability of ORNs to bring into being new synapses is due to the presence of special glial cells called Olfactory Ensheathing Cells (OECs), a particular glial type of the olfactory system (Graziadei and Monte-Graziadei, 1980). The embryonal origin of these cells is from the olfactory placodes of the ectoderm (Gómez et al., 2018). During their development, they migrate and accompany the non-myelinated axons of the ORNs towards the OB (Ramon-Cueto and Avila, 1998; Fairless and Barnett, 2005). These cells unsheath the olfactory nerve fibers and assist axon growth from the olfactory mucosa towards to the OB (Windus et al., 2011; Ekberg, and Amaya.D., Mackay-Sim, A., St John, J.A., 2012; Nazareth, Lineburg, Chuah, et al., 2015). The OECs guide the olfactory axons of the ORNs along the lamina propria, crossing the cribrosa plaque of the ethmoid bone, forming small bundles directed to the glomeruli inside the OB, where they synapse with the neurons of the olfactory nerve (Doucette, 1990; Nazareth, Lineburg, Chuah, et al., 2015). The role of the OECs is not to form myelin along

the olfactory axons but to group them together, acting as a mechanical support. (Barton, St John, Clarke, Clarke, and Ekberg, 2017) (Fig. 1). The interaction between olfactory axons and the OB is made possible by OECs through glutamatergic and purinergic pathways (Gòmez et al., 2018; Rieger, Deitmer, and Lohr, 2007). Moreover, several studies have demonstrated that OECs, transplanted into the damaged spinal cord, have the ability to promote the migration and regeneration of injured axons (Lu, Féron, Ho, Mackay-Sim, and Waite, 2001; Lu and Ashwell, 2002; Lu, Féron, Mackay-Sim, and Waite, 2002; Yang, He, and Hao, 2015). The OECs show dynamism in size and nature, they are able to change morphology in a short time (Sonigra, Brighton, Jacoby, and Wigley, 1999; Van den Pol and Santarelli, 2003), moreover, they also share properties with Schwann cells and astrocytes (Doucette, 1990; Mackay-Sim and Kittel, 1991; Wewetzer, Verdù, Angelov, and Navarro, 2002).

Immunocytochemistry and PCR studies have shown that OECs express adhesion molecules, such as laminin, fibronectin, neural antigen/glia 2 (NG2), and galectin-1, in addition to trophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophic factor derived from glial cells (GDNF) (Woodhall, West, and Chuah, 2001; Moreno-Flores, Díaz-Nido, Wandosell, and Avila, 2002); they play a role in regeneration (Franceschini and Barnett, 1996; Doucette, 1996; Kafitz and Greer, 1998; Moreno-Flores, Díaz-Nido, Wandosell, Avila, and Wandosell, 2003; Tan, Zhang, and Levine, 2005; Cao et al., 2010; Barton, St John, Clarke, Clarke, and Ekberg, 2017), as

well as their receptors TrkB, TrkC, GFRa-1 and GFRa-2 (Woodhall, West, and Chuah, 2001; Lipson, Widenfalk, Lindqvist, Ebendal, and Olson, 2003).

1.2. Ghrelin and its receptor in the olfactory system

Several studies suggest that Ghrelin (Ghre), an orexigenic peptide, plays an important role in all the olfactory system, and acts not only in olfactory cerebral areas, but also on the most peripheral part of the olfactory system (Tong et al., 2011; Trellakis et al., 2011; Okuhara, 2018). Ghre, a polypeptide of 28 amino acids, is a gut hormone produced in the stomach; it is able to reach the Central Nervous System (CNS) by regulating metabolism and neuroprotection (Banks, 2008; Bayliss et al., 2016).

The gene encoding Ghre has been identified in numerous species and its sequence is highly conserved among mammals (Angeloni et al., 2004; Perelló-Amorós et al., 2018). The human Ghre gene is located on chromosome 3 in position 3p25-26 and encodes a pre-pro-hormone of 117 amino acids, called pre-pro-Ghrelin. It is modified by protease cleavage and acylation in a bioactive peptide, through the post-translational addition of n-octanoic, and n-decanoic acids, by the action enzyme of Ghre-O-acyltransferase (GOAT), essential to activate its endogenous ligand for the GH secretagogue receptor (GHS-R 1a) (Kojima et al., 1999; Date et al., 2000; Van der Lely, Tschöp, Heiman, and Ghigo, 2004; Yang, Zhao, Goldstein, and Brown, 2008; Zaniolo et al., 2011).

GHS-R and Ghre mRNA are found in many tissues, both central and peripheral (Guan et al., 1997). On the synthesis of the Ghre peptide at the central level there is a controversy; some authors deny its synthesis or admit it in small concentrations not acknowledging its function (Cabral, López Soto, Epelbaum, and Perelló, 2017; Furness et al., 2011) while others clearly highlight it, particularly at the level of hypothalamic nuclei, such as arcuate, ventromedial, etc (; Lu et al., 2002; Sato, Fukue, Teranishi, Yoshida, and Kojima, 2005; Kohno, Sone, Minokoshi, and Yada, 2008; Ferrini, Salio, Lossi, and Merighi, 2009; Qi, Inoue, Fu, Inui, and Herzog, 2015; Takemi et al., 2016; Gong et al., 2017; Liu et al., 2019).

Our previous studies showed that OB mitral cells express Ghre, and play an important role in regulating feeding behavior in response to odors (Russo, Russo, Pellitteri, and Stanzani, 2018) (Fig. 2). Moreover, other authors evaluated the possibility that a pre-treatment of the olfactory epithelium with Ghre can modify the reactivity of olfactory neurons in the OB (Loch, Breer, and Strotmann, 2015). The binding between Ghre and its receptor increases intracellular calcium levels (Kaiya, Kangawa, and Miyazato, 2013). Ghre is able to pass through the blood-brain barrier, connecting the central and peripheral systems (Banks, 2008; Howick, Griffin, Cryan, and Schellekens, 2017). In fact, GHS-R 1a was also found in several brain regions including the hypothalamus, hippocampus, substantia nigra, and olfactory bulb and in other regions previously unknown to express GHS-R 1a mRNA (Mani et al., 2014; Rhea et al., 2018). Chronic administration of

acylated Ghre, through GHS-R 1a, might have mediated a protective effect in a mouse model of Parkinson's disease (Andrews et al., 2009). Exogenous acyl-Ghre reduces gliosis as an anti-inflammatory agent contributing to the neuroprotective effect inhibiting the release of some pro-inflammatory cytokines (Dixit et al., 2004).

The aim of this in vitro study was to identify and confirm the presence of the Ghrelin peptide and its GHS-R 1a receptor in OECs.

The survey was conducted through:

- Immunocytochemical procedures, to detect the presence of this specific protein and its receptor using specific antibodies binding to them;

- Western blot analysis, to identify the presence of this specific single protein and its receptor within a complex protein mixture. This allows a semi-quantitative estimation of the protein that can be obtained from the size and intensity of the color of a protein band on the membrane-stain;

- RT and qRT-PCR, for the detection of Ghre and GHS-R 1a mRNA. This method measures the mRNA levels after amplification, with highly reproducible results.

The presence of Ghre and its receptor in OECs, induced us to hypothesize on their involvement in a circuit that intervenes in the consolidation of the interaction between ORNs and Ghre-Mitral Cells, supporting their synaptic connection.

2. Materials and methods

2.1. Animal care

Experiments were performed on 2-day old rat pups (P2; Envigo RMS, Italy). Animals were kept in a controlled environment (23±1 °C, 50±5% humidity) with a 12 h light/dark cycle with food and water available ad libitum. Experiments were carried out in compliance with the Italian law on animal care no. 116/1992 and in accordance with the European Community Council Directive (86 / 609 / EEC). All efforts were made to minimize animal suffering and to use the fewest animals possible.

2.2. OEC cultures

OECs were isolated from 2-day old rat pup (P2) olfactory bulbs, according to our previous study (Pellitteri, Spatuzza, Russo, and Stanzani, 2007). Briefly, pups were decapitated and the bulbs removed and dissected in the cold (+4 °C) Leibowitz L-15 medium (Sigma).
Subsequently, they were digested in Medium Essential Medium-H (MEM-H, Sigma) containing collagenase and trypsin. Trypsinization was stopped by adding Dulbecco's Modified Eagle's medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Sigma).

Cells medium DMEM/FBS, 2mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). The antimitotic agent, cytosine arabinoside (10−5 M), was added 24 h after initial plating to remove fibroblasts; successively, OEC cultures were processed with an additional step of transferring cells from one flask to a new one (Chuah and Au, 1993).

This step reduces contaminating cells because they adhere more readily to plastic than OECs, consequently forming a cellular substratum upon which OECs are attached. Hence, during trypsinization, the OECs were the first cells to detach and if the enzymatic digestion was carefully monitored and stopped at this stage, this manipulation leaves most of the contaminating cells on the plastic. In the last passage, OECs were plated on 25 cm2 flasks and cultured in DMEM/FBS supplemented with bovine pituitary extract. Cells were incubated at 37 °C in DMEM/FBS and fed twice a week. When confluence was increased, OECs were replated onto 14-mm diameter poly-L-lysine (PLL; 10 μg/ml) coated glass coverslips at a final density of 1x104 cells/coverslip and fed with fresh complete medium.

2.3. Immunocytochemistry

After a 3-day culture, OECs were processed through immunocytochemical procedures. Cells were fixed by exposing them to 4% paraformaldehyde in 0.1 M PBS for 30 min. After washing in PBS the cell membranes were permeabilized with 5% normal goat serum (NGS) in PBS containing 0.1% Triton X-100 (PBS-Triton) at

room temperature for 15 min. To verify OEC purity, some coverslips were incubated overnight with polyclonal antibody anti-S-100 (1:400; Dako) and anti-p75 (rabbit, 1:500, Chemicon); they were successively incubated with the secondary Cy3 anti-rabbit antibody (1:200; Jackson ImmunoResearch, Laboratories, Inc.). The same method was applied for detection of Ghre and GHS-R 1a, using rabbit anti-ghrelin (1:200, Santa Cruz Biotechnology Inc.) and anti-ghrelin receptor type 1a (1:200, Santa Cruz Biotechnology Inc.) as primary antibodies. Successively OECs were incubated with the Cy3 anti-rabbit antibody (1:200; Jackson ImmunoResearch, Laboratories, Inc.). The cell nuclei were counterstained, for 10 min, with DAPI (4′,6-diamidino-2-phenylindole). The immunostained coverslips were analysed with a Nikon i80 fluorescence microscope, and images were captured with a Nikon Imaging System. No non-specific staining of cells was observed in control incubations, in which the primary antibody was omitted.

2.4. Western blot analysis

For immunoblotting, OECs were plated at a density of 1x104 cells/cm^2 in growth medium. Cells were cultured for 3 days under standard culture conditions, 5% CO_2 at 37°C. The day of the experiment, media were removed, cells were washed in PBS and incubated with trypsin (Sigma-Aldrich) for 3 min at 37 °C. Cells were then harvested and centrifuged at 300g for 5 min. Cell pellets were homogenized in 30 μl 1X Ripa Lysis Buffer (Abcam) supplemented with a cocktail of protease inhibitors (1:100, Sigma-Aldrich). The total protein content

was quantified and 50 μg of protein were electrophoresed on 4-20% SDS-Page gel and transferred to nitrocellulose membranes. After blocking with Odyssey blocking solution (LI-COR Biosciences) for 1 h at room temperature, membranes were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-Ghre (1:200, Santa Cruz Biotechnology Inc.), rabbit anti-GHS-R 1a (1: 200, Santa Cruz Biotechnology Inc.) and mouse anti-actin (1:5000, Abcam). The day after, membranes were washed 3 times in PBS-0.1% Tween for 5 min and incubated for 1 h at room temperature with appropriate secondary antibodies: IRD800 or IRD700 goat anti-mouse (1:10000, LI-COR) and IRD800 or IRD700 goat anti-rabbit (1:10000, LI-COR).

Proteins bands were detected using an ODYSSEY infrared imaging system (LI-COR Biosciences).

2.5. Ghrelin PCR analysis

2.5.1. RNA isolation and reverse transcription (RT)

As previously described (Barresi et al., 2018), total RNA was extracted from the cell line using the RNeasy Mini kit (cat no. 74104, Qiagen, Germany) following the manufacturer's instructions. RNA concentration and quality were assessed using the ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, USA). We reverse transcribed (RT) 2 μg of total RNA by SuperScript III First-Strand Synthesis SuperMix kit using random hexamers according to the manufacturer's instructions (Invitrogen, USA).

2.6. PCR Primer Design and qualitative PCR

Primers used for qualitative PCR were designed by the "Primers-BLAST" tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primerblast/) using the rat ghrelin transcript sequence (rGHRE; Gene ID: 59301; NM_021669.2) and rat GH-secretagogue receptor (rGHSR, GHSR; Gene ID: 84022; NM_032075.3) as templates. The following primers were designed: rGHRE ex 1-2F (CCAGAAAGCCCAGCAGAGAA) and rGHRE ex 3R (ACTGAGCTCCTGACAGCTTG) whose product is 176 bp; rGHRE ex 1F (CTCAGCATGCTCTGGATGGA) combined with the previous primer rGHRL ex 3R that produces a fragment of 168 bp.

To detect the rGHS-R transcript we designed the following primers: rGHSR-1F 551 (AGCACGAAAACGGCACAGA) and rGHS-R-2R 869 (AGCGATCTCCAGAGAGCCAG) that amplifies a product of 338 bp. We confirm the rGHS-R amplification using the primers reported previously by Tena-Sempere et al., 2001: GHS-R sense (AGGCAACCTGCTCACTATGCTG) and GHS-R antisense (GACAAGGATGACCAGCTTCACG), product size 321 bp.

Amplification was performed with GeneAmp PCR System 9700 (Applied Biosystem, USA) using Platinum Taq DNA polymerase (Invitrogen, USA) in a final volume of 50 µl including 200 ng of cDNA. As positive control, the expression level of the rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH; Gene ID: 24383; NM_017008.4) was determined using the following primers: GAPDH 3F

(GTCAAGGCTGAGAACGGGAA) GAPDG 45R (TGAGCCCCAGCCTTCTC), PCR product is 155 bp. Thermal conditions required for the reaction were a preheating step at 94 °C (30 sec), followed by 45 cycles of 94 °C (30 sec), 60 °C (30 sec) and 72 °C (1 min), and a final cycle of extension at 72 °C (5 min). PCR products were separated by 1.8 agarose gel electrophoresis.

All PCR products were column-purified with HiYield Gel/PCR DNA Fragment Extraction Kit (RBC BIOSCENCE, Taiwan) and sequenced by the standard dideoxy chain termination procedure with the Abi Prism 377 automatic sequencer in both directions (Barresi et al., 2010).

2.7. Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) was performed using StepOneTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following primers: GHS-R ex 1F/ex 3R and GHS-R ex 1F/ex 2R; the reaction was performed on 200 ng of cDNA, as mentioned above, using SYBR GREEN PCR Master Mix (Applied Biosystem, Foster City, CA, USA). As previously reported in Barresi et al., 2018 each sample was analysed in triplicate and the average was normalized to rat GAPDH expression. Amplification conditions included a cycle at 95 °C for 10 minutes followed by 45 cycles at 95 °C for 15 seconds and a cycle at 60 °C for 1 minute. As a negative control, reaction without cDNA was performed (no template control, NTC). The relative RNA expression level for each sample was calculated using the 2-ΔΔCT method.

3. Results

3.1. Characterization and Morphological Features of OECs

Cellular morphology was monitored by phase-contrast microscopy (Fig. 3A), while cell purification was verified using the immunocytochemical technique with antibodies recognizing specific protein markers for OECs, such as S-100 protein and p75 (Fig. 3 B-C). These analyses showed that, after purification, the majority of OECs exhibited both star and spindle shapes, which are typical morphological features of OECs.

3.2. Immunocytochemical expression of Ghre and GHS-R 1a in OEC cultures

Our results, we believe for the first time, demonstrate the existence of Ghre protein and its receptor in primary OECs, using immunocytochemical analysis, with Ghre and GHS-R 1a antibodies. As part of the immunocytochemical analysis, the use of a negative control excluded the non-specific immunoreactivity of OECs to both the antighrelin antibody and the GHS-R 1 receptor, in fact, no labeling was observed when the primary antibodies were omitted. We found that OECs were able to express both Ghre and GHS-R, as reported in Figure 4, which shows a representative field from the immunostained OECs to 3 days of culture with anti-ghrelin antibodies (A-B) and anti-GHS-R 1a (C-D).

3.3. Western Blot analysis

In order to highlight whether OECs express Ghre and its receptor GHS-R 1a, we performed a Western blot analysis of the total protein contents of OEC cultures after 1 and 4 passages post-derivation. We found that OECs express Ghre and GHS-R 1a and that these proteins were detectable after extensive passages in vitro (Fig. 4, E); this finding is consistent with the expression of these proteins at the mRNA level. No differences were found between the two time-points, for either proteins as compared to the corresponding actin levels.

3.4. PCR analysis

The results obtained on the OECs, with the immunocytochemical technique and Western Blot, regarding the expression of Ghre and its receptor, are were further confirmed by the Polymerase Chain Reaction (PCR).

3.4.1. Rat Ghre-mRNA is expressed in OECs

The transcript of Ghre comprises four exons (Ex1-Ex4), thus two primer pairs were designed and used to detect Rghre-mRNA: the first forward primer encompassed the junction between exon 1 and exon 2 (Ex1 and Ex2, forward), while the reverse primer was designed on exon 3 (Ex3, reverse); the second couple was designed on exon 1 (Ex1, forward) and on exon 3 (Ex3, reverse). As shown in Figure 5, PCR products with the predicted size and longer size were obtained, thus all the amplifiers were purified and sequenced. Ovarian tissue was used as

that the smaller products (176 and 168 bp) corresponded to nativeghrelin, while the longer ones (270 bp) corresponded to unspecific products (Fig. 5, A).

qRT-PCR revealed that native-ghrelin (Ghrel) is detected at lower levels in OECs compared with rat intestine, hippocampus and hypothalamus. Intestine was used as a positive control, with a Foldchange < -1.5 (Figure 6 A). We also reported ΔCTvalues as 1/ΔCT in order to show that Ghrel expression has been detected and normalized versus GAPDH in all samples. (Figure 6 B).

3.4.2. Rat GHS-R1-mRNA is expressed in OECs

PCR amplification of the transcript encoding rGHS-R was detected in OECs using the primer couple rGHSR-1F 551 and rGHRS-2R 869, and confirmed with the following primers: GHS-R sense and GHS-R antisense. Figure 5 B compares the expression of OEC GHS-R with that already acquired in different areas of CNS and peripheral district in the rat. Ovarian tissue was used as negative control (data not shown). qRTPCR revealed that GHS-R, like its ligand rGhrel, is detected at lower levels in OECs compared with rat intestine, hippocampus and hypothalamus.

Hypothalamus was used as a positive control, with a Foldchange < -2.5 (Figure 6 C).

We also reported ΔCTvalues as 1/ΔCT in order to show that GHS-R expression has been detected and normalized versus GAPDH in all samples (Figure 6 D). This highlight, unequivocally for the first time,

the expression of GHS-R in this particular type of olfactory cells, thus expanding potential expectations regarding its use.

4. Discussion

It is now known that the immune-reactive Ghre (Ghre-IR) signal, in addition to being found at the level of the various peripheral organs, is also present centrally; although some authors do not consider the quantity produced, centrally, of Ghre sufficient to have a physiological meaning (Cabral, López Soto, Epelbaum, and Perelló, 2017; Furness et al., 2011), indeed, many authors, using antibody-based detection can be synthesized at the CNS level (Lu and Ashwell, 2002; Sato, Fukue, Teranishi, Yoshida, and Kojima, 2005; Kohno, Sone, Minokoshi, and Yada, 2008; Ferrini, Salio, Lossi, and Merighi, 2009; Qi, Inoue, Fu, Inui, and Herzog, 2015; Takemi et al., 2016; Gong et al., 2017; Liu et al., 2019).

It has also been shown that the use of the Ghre receptor antagonist is able to block the effect of Ghre itself, highlighting the role of the peptide and its receptor in neuronal reactivity (Loch, Breer, and Strotmann, 2015). The region of the brain with the highest level of the Ghre peptide, regardless of species, is the olfactory bulb, in which there is the highest absorption of human Ghre with a radio-labeled peripheral injection in the mouse brain (Diano et al., 2006; Rhea et al., 2018). Furthermore, the presence of GHS-R 1a in olfactory circuits has been demonstrated allowing Ghre to improve the ability to search for food through smell; increasing olfactory sensitivity (Tong et al., 2011). At the level of the

olfactory mucosa, ORNs, along the axons of the nervous layer, release glutamate and ATP by exocytosis (Lohr, Grosche, Reichenbach, and Hirnet, 2014), binding, in the outer nervous layer, to the mRluR1 and P2Y1 receptors of OECs, respectively (Fig. 7).

The Ghre produced by Mitral Cells (MC), interact with the GHS-R 1a expressed on the OECs and stimulate the same cells (OECs) to secrete Ghre, which, in turn, would intervene on the axon of ORNs. Moreover, it is known that Ghre, by secreting factors (Yang et al., 2013), has a neurotrophic action both on promotion of axonal growth of neurons and on synaptogenesis (Stoyanova and Le Feber, 2014), we can hypothesize that also OECs, through Ghre, can improve the growth of neurites and promote their alignment (Khankan, Wannerm, and Phelpsm, 2015).

In our previous work, we highlighted how Ghre can also act on the most peripheral part of the olfactory system (Russo, Russo, Pellitteri, and Stanzani, 2018): its activity, in fact, has been demonstrated on the sensory olfactory receptors at the level of the main olfactory epithelium (MOE) through the binding to its GHS-R 1a (Loch, Breer, and Strotmann, 2015; Tong et al., 2011; Trellakis et al., 2011). Since it has been shown that OECs are a source of trophic factors (Pellitteri, Spatuzza, Stanzani, and Zaccheo, 2010), they are able to communicate with their environment by virtue of these trophic molecules (Radtke and Kocsis, 2012) and therefore have the possibility of neuronal protection and can promote the growth of axons: OECs in vitro are able to express these factors and promote axonal lengthening (Woodhall, West, and Chuah, 2001). In vitro, in a co-culture model, OECs migrated along the

neurons of the organotypic brain stem slices, thus eventually helping axonal guidance (Jiao, Novozhilova, Karlén, Muhr, and Olivius, 2011), allowing the continuous growth and synaptogenesis of the olfactory axons in the CNS (Pastrana et al., 2006).

In the present study, we hypothesize that Ghre expressed by OECs can exert effects on the peripheral olfactory circuit activating the relationship between ORN and MC. Our results allow us to hypothesize the consolidation of a circuit, in which Ghre, expressed by OECs, could contribute to the synaptic interaction of ORN-MC. Therefore, it is very likely that, along the circuit described (Figure7), an increase in the sensitivity expressed in ORNs can be mediated by the action of Ghre and its receptor.

In conclusion, our study demonstrates that Ghre and GHS-R 1a transcripts are expressed by OECs, moreover, our results support, for the first time, the hypothesis that these cells, through the secretion of Ghre, can interact, with a reinforcement function, in the peripheral olfactory circuit, providing a neurotrophic support to the synaptic interaction between olfactory neurons and mitral cells.

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Declaration of Competing Interests

The authors declare that they have no conflict of interest.

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Figure 1

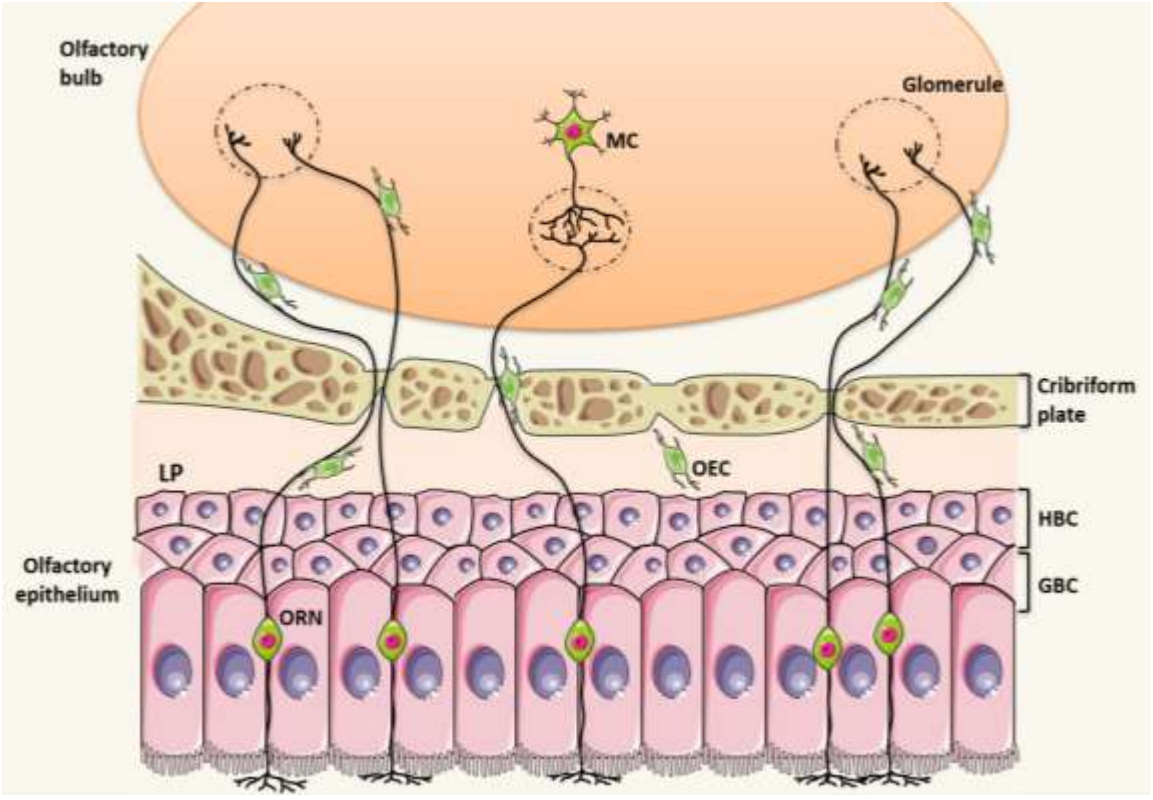

Fig. 1. A schematic representation of the olfactory system. Olfactory epithelium: ORN = Olfactory Receptor Neuron; GBC = Globose Basal Cells; HBC = Horizontal Basal Cells; LP= Lamina Propria; OECs = Olfactory Ensheathing Cells; MC = Mitral Cell. The figures were produced using Servier Medical Art.

Figure 2

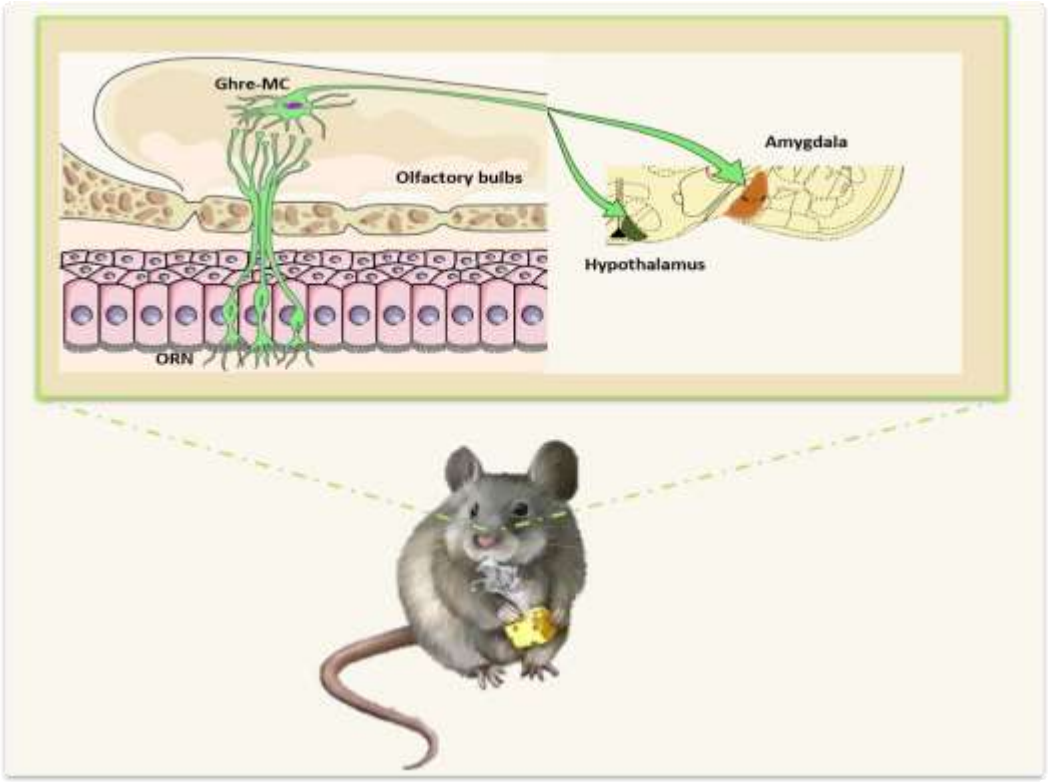

Fig. 2. Projection of Mitral Cells. The figure shows a schematic view of our proposed mechanism, for which Ghre-MC (ghrelin- mitral cell) of the olfactory bulbs, that receive information from the ORN (olfactory receptor neuron) might promote food intake responding to odors involving hypothalamus and amygdala

Figure 3

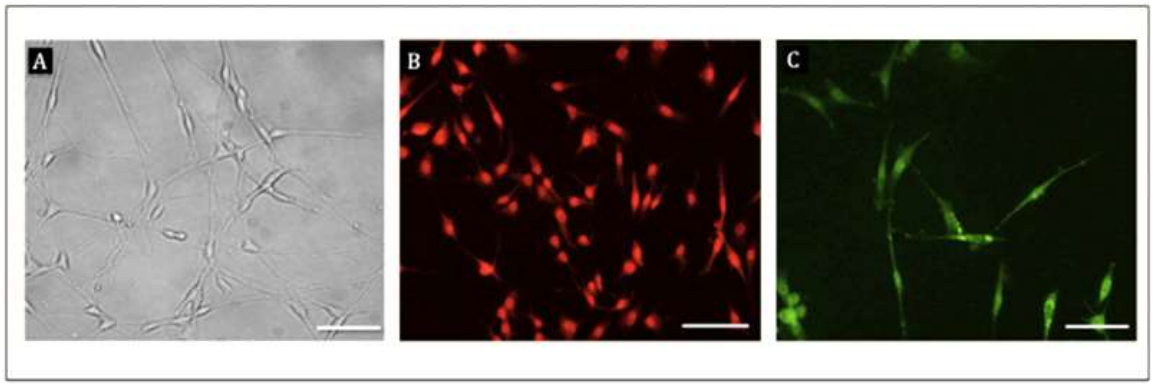

Fig. 3. Morphological features of OECs. Qualitative analysis by phase-contrast microscopy (A), immunostained with S-100 protein antibody (B) and P75 (C) of representative fields of OECs (20X). Scale bars: 50μm

Figure 4

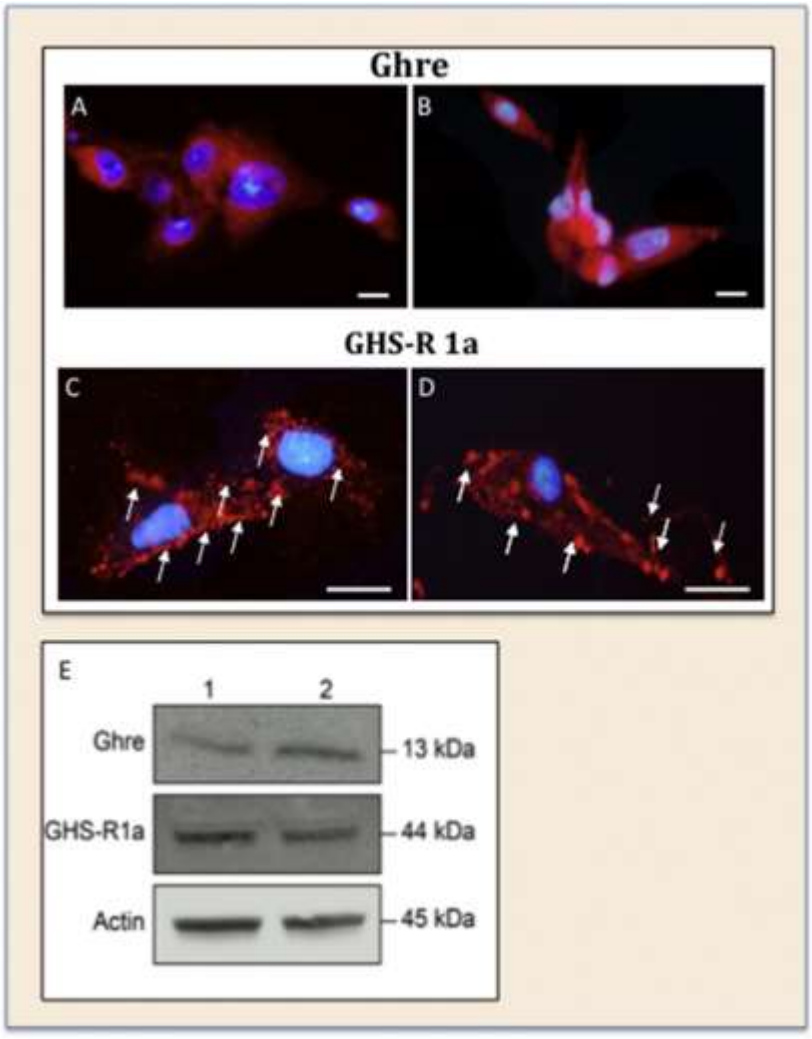

Fig. 4. Expression of Ghre and GHS-R 1a in OECs. (A-D) representative fields of immunostained OEC 3-day cultures stained with anti-Ghre (1:200) (A-B) or anti-GHS-R 1a (1:200) (C-D) antibodies. Scale bars: 20μm; (E) representative western blot detection of Ghre and GHS-R 1a in OEC cultures at passage 1 (lane1) and passage 4 (lane 2) after derivation. C. Russo, et al. Neuropeptides 79 (2020) 1019975

Figure 5

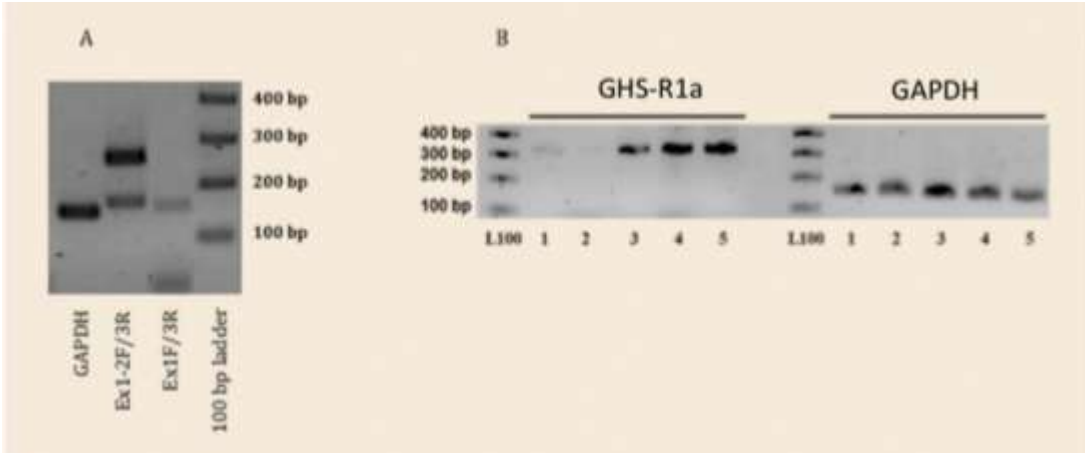

Fig. 5. *Polymerase Chain Reaction (RT-PCR) analysis.* In (A) RT-PCR amplification obtained in rat OECs using the GHS-R ex 1-2F and GHS-R ex 3R (176 bp) and GHS-R ex1F combined with previous primer GHS-R ex 3R (168 bp). 270 bp PCR product is unspecified. In (B) RT-PCR amplification of the rGHSR transcript (321 bp) and GAPDH (155 bp) as control in rat intestine (1), rat hippocampus (2), rat hypothalamus (3), OECs (4), OECs (5). L100: ladder 100 bp.

Figure 5

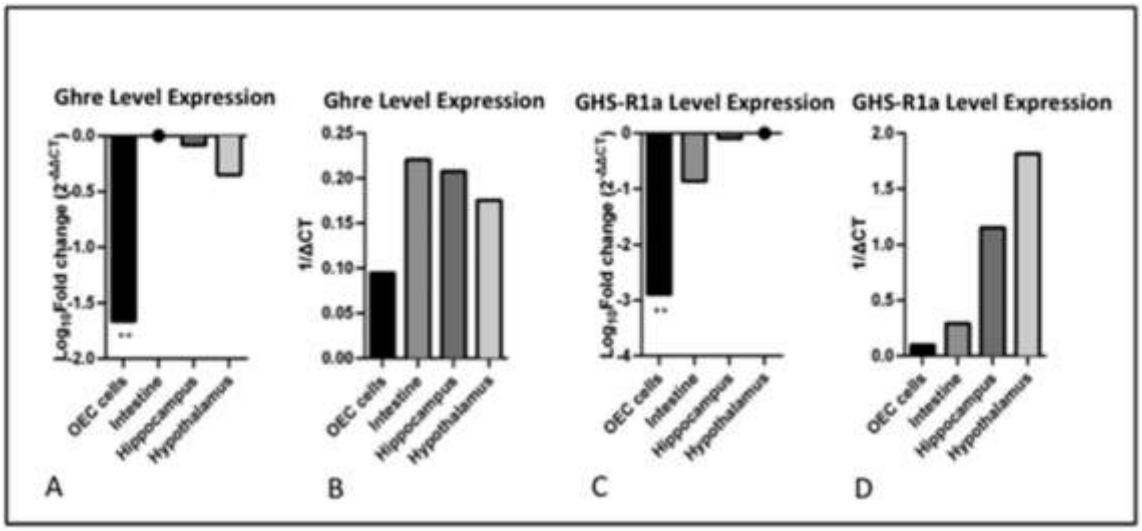

Fig. 6. *Ghre and GHS-R expression levels*. Ghrelin (A,B) and GHS-R (C,D) transcript levels were measured by qRT-PCR in OECs, intestine, hippocampus and hypothalamus, and expressed as "log10Fold change (2-ΔΔCT)" in A,C and as "1/ΔCT" in B,D. Statistical differences were assessed using Student's t-test. **p <0.001.

Figure 7

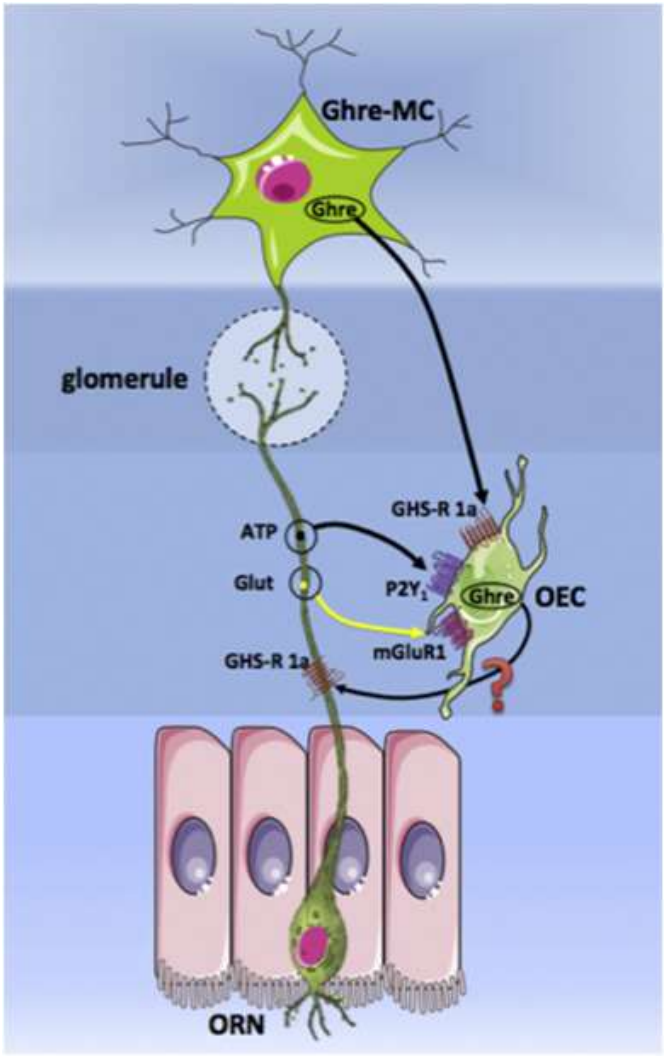

Fig. 7. *The axon-glia interaction between the epithelium and the bulb within the olfactory system*. This drawing schematizes it, which can be mediated by the OECs through the peptide ghrelin. Ghre- MC = ghrelin mitral cell; Ghre = ghrelin; GHS-R 1a = Ghrelin Receptor; P2Y1 = Purinoceptors; mGluR1 = glutamate receptor; OEC = Olfactory Ensheathing Cell; ATP = adenosine 5′-triphosphate; Glut = glutamate; ORN = Olfactory Receptor Neuron; MCL = Mitral Cell Layer; GL = Glomerular Layer; ONL = Outer Nerve Layer; MOE = Main Olfactory Epithelium; OEL = Olfactory Epithelium Layer.

Effects of Ghrelin on Olfactory Ensheathing Cell Viability and Neural Marker Expression

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Abstract

Ghrelin (Ghre), a gut–brain peptide hormone, plays an important role in the entire olfactory system and in food behavior regulation. In the last years, it has aroused particular interest for its antioxidant, anti-inflammatory, and anti-apoptotic properties. Our previous research showed that Ghre and its receptor are expressed by peculiar glial cells of the olfactory system: Olfactory Ensheathing Cells (OECs). These cells are able to secrete different neurotrophic factors, promote axonal growth, and show stem cell characteristics. The aim of this work was to study, in an in vitro model, the effect of Ghre on both cell viability and the expression of some neural markers, such as Nestin (Ne), Glial Fibrillary Acid Protein (GFAP), Neuregulin (Neu), and β-III-tubulin (Tuj1), in primary mouse OEC cultures. The MTT test and immunocytochemical procedures were used to highlight cell viability and marker expression, respectively. Our results demonstrate that Ghre,

after 7 days of treatment, exerted a positive effect, stimulating OEC viability compared with cells without Ghre treatment. In addition, Ghre was able to modify the expression of some biomarkers, increasing Neu and Tuj1 expression, while GFAP was constant; on the contrary, the presence of positive Ne cells was drastically reduced after 7 days, and this showed a loss of stem cell characteristic and therefore the possible orientation towards an adult neural phenotype.

Keyword

Ghrelin, Olfactory Ensheathing Cell cultures, Immunocytochemistry, Neural phenotype, Trophic effect

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Introduction

Ghrelin (Ghre) is a small peptide hormone, known for its role in appetite stimulation, feeding behavior, energy homeostasis, and carbohydrate metabolism (Cowley et al. 2003; Muller et al. 2015). It contributes, through the interaction with its receptor, in a large number of functions from typical orexigenic action to the role on the brain pathways (Russo et al. 2017; Young and Jialal 2019). Ghre plays a role in the regulation of neuroendocrine and neurodegenerative processes, especially in higher brain functions, such as learning and memory consolidation (Panagopoulos and Ralevski 2014; Jiao et al. 2017), providing a neurotrophic support to the synaptic interaction between olfactory neurons and mitral cells (Russo et al. 2018).

The role of Ghre can be related to synaptic formation and neurogenesis. A recent report shows that Ghre stimulates synaptic formation in cortical pathways in cultures improving synapse recovery in an in vitro model (Stoyanova et al. 2016); in vivo, it promotes cortical neurite growth (Liu et al. 2019) and stimulates adult hippocampal neurogenesis for learning and memory (Kent et al. 2015), reorganizing the dendritic spines of the hippocampal neurons and promoting their regeneration, through the continuous activation of its receptor (Berrout and Isokawa 2012).

Olfactory Ensheathing Cells (OECs), in situ, appear with an elongated shape and a thin laminar process surrounding the olfactory nerves, while in vitro, their morphology is characterized by a flat, bipolar, or multipolar appearance (Franceschini and Barnett 1996; Vincent et al.

2005). It has been shown that OECs secrete neurotrophins (NT) and other trophic factors capable of promoting neurite elongation of olfactory neurons in vitro (Kafitz and Greer 1999). Other studies have demonstrated that the OECs express, also in vitro, neurotrophic factors, such as Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophic Factor Derived from glial cells (GDNF), neurturin (NTN), and neuregulins (Woodhall et al. 2001; Moreno-Flores et al. 2002; Pastrana et al. 2007), as well as their receptors TrkB, TrkC, GFRa-1, and GFRa-2 (Woodhall et al. 2001), promoting neuron survival and axonal growth (Huang and Reichardt 2001; Pellitteri et al. 2007; Pellitteri et al. 2009). In addition, OECs also promote angiogenesis, migration, and interaction with scar tissue as well as spinal cord remyelination (Franssen et al. 2007; Pellitteri et al. 2010; Ramón-Cueto et al. 2000). They contribute to the regeneration of the primary olfactory nervous system and, with their regenerative potential, they play an important role in transplants in the injured spinal cord (Franssen et al. 2007). Moreover, they express adhesion molecules and numerous markers, such as Glial Fibrillary Acidic Protein (GFAP), p75NTR, S100, vimentin, and nestin (Ramón-Cueto and Avila 1998; Pellitteri et al. 2010), that can be recognized by immunostaining in vitro and in vivo (Alexander et al. 2002.; Vincent et al. 2005; Gómez et al. 2016). In particular, p75NTR and S100 expressions were used to identify OECs (Moreno-Flores et al. 2003; Pellitteri et al. 2009). In addition, OECs are positive to nestin in embryonic and postnatal stages (Ramón-Cueto and Avila 1998), while expressing GFAP in the

postnatal and adult stages (Barber and Dahl 1987). Some data highlight that a variable expression of β-III-tubulin (Tuj1) is present in several glial cell types, suggesting that this cell population has the potential to differentiate into both neurons and glia (Itoh et al. 2006; Rieske et al. 2007).

Our recent study also demonstrated the OEC ability to express Ghre and its receptor. This evidence suggests that Ghre could act both in a paracrine and autocrine manner on the OEC expression of some neuropeptides and other secretion factors involved in the processes of neuroprotection, synaptogenesis, and neurodifferentiation (Russo et al. 2020).

Therefore, the purpose of this work was to study the effect of Ghre on some neural markers, such as nestin (Ne) as a stem marker, GFAP expressed exclusively by glial cells, neuregulin (Neu) as neuronal growth factors and axonal myelination, and Tuj1, to visualize potential orientation towards a neuronal phenotype in OECs through immunocytochemical procedures. In addition, we evaluated the cellular viability using the MTT test.

Materials and methods

Animals

Experiments were performed on 2-day-old mice pups (P2; Envigo RMS, Italy). Animals were kept in a controlled environment (23 ± 1 °C, 50 ± 5% humidity) with a 12-h light/dark cycle with food and water available ad libitum. All the experimental procedures were carried out

according to the Italian Guidelines for Animal Care (D.L. 116/92 and 26/2014), which are in compliance with the European Communities Council Directives (86/609/EEC) and were approved by the Ethical Committee of the University of Catania (Organismo Preposto al Benessere Animale, OPBA; Authorization n. 174/2017-PR). All efforts were made to minimize animal suffering and to use the fewest number of animals possible.

OEC cultures

OECs were isolated from olfactory bulbs of 2-day-old mice pups (P2) as described by Pellitteri et al. (2007). After pup decapitation, the bulbs were removed, dissected out in cold (+ 4 °C) Leibowitz L-15 medium (Sigma), and digested with collagenase and trypsin added to medium essential medium-H (MEM-H, Sigma). To stop trypsinization, Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Sigma) was used. Cells were resuspended and then plated in flasks with fresh complete medium DMEM/FBS, and antibiotics (Sigma). After 24 h from the first plating, cytosine arabinoside (10–5 M, antimitotic agent; Sigma) was added for 48 h to reduce the number of dividing fibroblasts. The purification procedure of Chuah and Au (1993) was used to reduce the number of contaminating cells. The percentage of p75/S-100-positive cells in our cultures was about 90–95% (data not shown). In the last passage, OECs were plated on 25-cm2 flasks and cultured in DMEM/FBS supplemented with bovine pituitary extract (Sigma).

Treatment of OECs

Purified OECs were replated on 14-mm-diameter poly-L-lysine (PLL, 10 μg/ml, Sigma)–coated glass coverslips at a final density of 0.3 × 104 cells/coverslip and grown in DMEM/FBS (Sigma). In some cultures, 24 h post-seeding, Ghre (Abcam), reconstitute by adding deionized water, was added to the culture medium. We tested different concentrations of Ghre (data not shown), but we found that the optimal concentration was 2 μM, in accordance with previous report (Stoyanova et al. 2013). Control cultures (CTR) were fed with DMEM/FBS. Both of the experimental cell cultures, with and without treatment, were grown at different times (1, 3, 7 days) and incubated at 37 °C in a humidified 5% CO2–95% air mixture. All cultures were fed with DMEM/FBS; in particular, in those treated with Ghre at 7 days, the peptide was replenished for the second time after 3 days; successively, immunocytochemical procedures were assessed. To evaluate cellular viability by MTT, OECs were plated in 96 multiwells, incubated at 37 °C and grown both with and without Ghre treatment, following the same experimental protocol of the cells grown on 14-mm-diameter poly-L-lysine–coated glass coverslips.

MTT Bioassay

At the end of the Ghre treatment time, cellular viability survival was evaluated by the 3-[4,5-dimethylthiazol- 2-yl)-2,5-diphenyl] tetrazolium bromide (MTT, Sigma) reduction assay, a quantitative

colorimetric method. Briefly, MTT was added to each multiwell with a final concentration of 1.0 mg/ml and placed for 2 h in a CO2 incubator. Media were gently removed and MTT solvent (acid-isopropanol/SDS) was added; cells were then placed on an orbital shaker for 15 min. The absorbance was read by a multiskan reader at 570 nm.

Immunocytochemical procedures

The OECs were processed through immunocytochemical procedures. Cells were fixed by exposing them to 4% paraformaldehyde in 0.1 M PBS for 30 min. After washing in PBS, the cell membranes were permeabilized with 5% normal goat serum (NGS) in PBS containing 0.1% Triton X-100 (PBS-Triton) at room temperature for 15 min. OEC cultures were incubated overnight with the following neural markers: rabbit anti-GFAP polyclonal antibody (1:1000, Dako), rabbit anti-nestin polyclonal antibody (1:200, Immunological Sciences), rabbit anti-neuregulin polyclonal antibody (1:100, Santa Cruz Biotechnology, INC.), and mouse anti-Tuj1 monoclonal antibody (1:200 Abcam). Successively, Cy3 anti-rabbit (1:500, Immunological research) and Cy3 anti-mouse (1:500, Immunological research) were used as secondary antibodies for 1 h at room temperature and in the dark. The cell nuclei were counterstained for 10 min with DAPI (4′, 6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to adenine–thymine–rich regions in DNA.

The immunostained coverslips were analyzed with a Nikon i80, fluorescence microscope, and images were observed with a × 20

objective lens and captured using a Nikon Imaging System. No non-specific staining of cells was observed in control incubations in which the primary antibody was omitted.

Data analysis

For the MTT assay, a minimum of four experiments were carried out for each culture condition. Results were expressed as the percentage with respect to the values obtained for MTT reduction by OECs-CTR at 1 day. Differences between culture treatment were assessed using one-way analysis of variance (one-way ANOVA) followed by the post hoc Holm–Sidak test. Values for each group were expressed as the mean ± SD. Differences were considered statistically significant at $p < 0.05$.

The quantification of the cellular mean fluorescence was determined from six random fields per coverslip to highlight the time trend of the individual experimental groups, and also to compare the difference between OECs-CTR and OECs-Ghre treated. The fluorescence microscope images in the ImageJ software (or Fiji) were used, and a background correction was made (Abramoff et al. 2004). Three positive selections were chosen randomly around the cells to subtract the background intensity from the intensity measured in the pixels of interest.

Six independent images were obtained from each individual coverslip, with three coverslips per experimental condition. The relative fluorescence for each image was calculated by dividing the integrated intensity by the number of cells present in the field. The value for each of the three experiments represents the average of the values for the six images, and this value is expressed as mean percentage intensity of fluorescence/cell. For Fiji statistical evaluation, we used one-way ANOVA followed by the post hoc Holm–Sidak test. A probability of $p < 0.05$ was considered statistically significant.

Results

Effect of Ghre on Cellular Viability

The effect of Ghre on OECs was performed using the MTT assay at 1, 3, and 7 days. The results showed that only after 7 days of treatment with the Ghre-enriched medium, there was a positive effect on cell growth and a significant increase ($p < 0.05$) of OECs compared with the CTR (Fig. 1a). In order to control cellular well-being, the cells were monitored by means of phase contrast microscopy (Fig. 1b).

Immunocytochemical Expression of Neural Markers in OECs

Immunocytochemistry and fluorescence quantification were used to evaluate some neural markers on OECs: Ne, Neu, GFAP, and TuJ1 were tested at 1, 3, and 7 days of culture. In particular, two conditions were investigated: OECs grown in DMEM/FBS with no Ghre treatment

(CTR) and OECs grown with Ghre treatment. Qualitative and quantitative data were collected from four independent experiments, one for each marker. The percentage of mean fluorescence was obtained through immunocytochemical procedures (ImageJ-Fiji) evaluating positive OECs with respect to total cell number (DAPI-positive cells) for each marker during the 7 days of treatment. The different markers are immunocytochemically tested and shown in the Figs. 2, 3, 4, and 5.

Immunolabeling of Nestin in OECs

Our results show that during the entire time course of culture treatment, the mean percentage intensity of Ne fluorescence/cell decreased significantly both in OEC-CTR and OEC-Ghre (Fig. 2A). On the seventh day, the presence of positive Ne cells was drastically reduced (Fig. 2B). Moreover, values for the CTR were significantly higher compared with the OECs treated with Ghre ($p < 0.05$). Figure 2C shows representative images of the Ne immunoreactivity in the OEC-CTR (Fig. 2C: b, d, f) compared with the OEC-Ghre (Fig. 2C: b′, d′, f′).

Immunolabeling of Neuregulin in OECs

As shown in Fig. 3A, the mean percentage intensity of Neu fluorescence/cell decreased at 7 days. In addition, the comparison between the Neu mean fluorescence in OEC-Ghre is higher than OEC-CTR (Fig. 3B) and shows a statistically significant difference ($p < 0.05$).

Figure 3C shows representative images of Neu immunoreactivity that is more expressed in OECs treated with Ghre (Fig. 3C: b′, d′, f′) than the OECs-CTR (Fig. 3C: b, d, f) during the time course. On the seventh day, the OEC-Ghre expressed low Neu in more differentiated cells (Fig. 3C: f′).

Immunolabeling of GFAP in OECs

During the time course cultures (1–3–7 days), the OECs maintained constant GFAP positivity, both in the controls and in those cells treated with Ghre. Therefore, Ghre does not change the expression of GFAP in the treated OECs (Fig. 4A, B, C).

Immunolabeling of Tuj1 in OECs

Our results show that Tuj1 immunoreactivity decreased in the OEC-CTR from the first to the seventh day. In fact, on the seventh day, it was drastically reduced (Fig. 5A). Instead, the treated OEC-Ghre maintained a high immunoreactivity that remained constant over time; it is evident that there is a clear increase of TuJ1 immunoreactivity in OEC-Ghre with respect to the OEC-CTR, particularly on the seventh day (Fig. 5B). Figure 5C shows representative images of TuJ1 immunoreactivity that is increased in OECs treated with Ghre (Fig. 5: b′, d′, f′) than the OEC-CTR (Fig. 5: b, d, f) during the time course. On the seventh day of Ghre treatment, the immunocytochemical analysis highlighted long extensions that might indicate a morphological modification (Fig. 5C: f′).

Discussion

It is known that OECs, being a source of multiple factors, play a decisive role in several processes such as regeneration and remyelination, and they could be used as trophic support and potential therapeutic agent (Gómez et al. 2018).

In fact, several studies, on animal models, concerning the implantation of OECs in the region of the damaged spinal cord, have demonstrated how they could intervene in axonal regeneration, remyelination, and functional recovery of spinal cord injury. Therefore, OECs could influence axonal growth stimulation, angiogenesis, migration, and interaction with scar tissue remyelination of the spinal cord (Gómez et al. 2018).

Recent studies on experimental models, both in vivo and in vitro, have shown how treatment with Ghre could promote axonal and dendritic growth through its neuroprotective action related to synaptogenesis and neurogenesis (Stoyanova et al., 2013; Stoyanova et al. 2016; Liu et al. 2019).

Some authors reported that Ghre could act as a neurogenic agent, favoring cell cycle progression through the mTOR pathway, and could also be involved in various cell differentiation processes (Chung and Park 2016). Moreover, Ghre, through the link with its receptor, could thereby intervene on the growth and differentiation pathways of OECs towards a neural phenotype.

In the present paper, the effect of Ghre on the expression of some biomarkers on OECs has been highlighted. In order to verify the state

of differentiation, Ne, a stem cell marker, was used. The ability of OECs to express Ne provides the possibility to evaluate the loss of their stem feature and the acquisition of a neural phenotype. It is known that once cellular differentiation is obtained, Ne is downregulated and replaced by cell-specific intermediate filaments, such as neurofilaments and GFAP (Halliday et al. 1996). Overall, our results highlight that the expression of Ne appears to be considerably lower following the treatment of OECs with the Ghre, thus indicating its gradual loss of stem cell characteristics and therefore the possible orientation towards an adult phenotype. The expression of some neural markers, such as Neu, GFAP, and Tuj1, was also tested, in order to visualize the potential orientation towards a neural phenotype.

Neu is involved in myelination processes, as modulator of the axonal myelin sheath formation (Belin et al. 2019), and in synaptic plasticity (Agarwal et al. 2014). Moreover, data support that Neu has a functional role in OECs (Thompson et al. 2000); GFAP is a component of the cytoskeleton of astrocytes and is also functionally involved in their plasticity and in their interactions with neurons (Pekny et al. 2007), and it is used as a glial differentiation marker of OECs (Barber and Dahl 1987); Tuj1 is present at all stages of neuronal differentiation, from some mitotically active neuronal precursors to differentiated neurons (von Bohlen unh Halbach 2007). Some authors showed the presence of high-level expression of Tuj1 in OECs, justifying the characteristic

properties of this type of cells, such as myelination, migration, and phagocytosis (Omar et al. 2013).

Therefore, in our study, we demonstrated that on the seventh day of OEC treatment with Ghre, Neu and TuJ1 were more expressed than OEC growth without Ghre treatment.

In conclusion, we can hypothesize that Ghre has a positive effect on OEC growth, thus it might be a promising candidate that is able to modulate the expression of OEC biomarkers acting both in a paracrine and autocrine manner.

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Conflicts of interest

The authors declare that they have no conflict of interest

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Figure 1

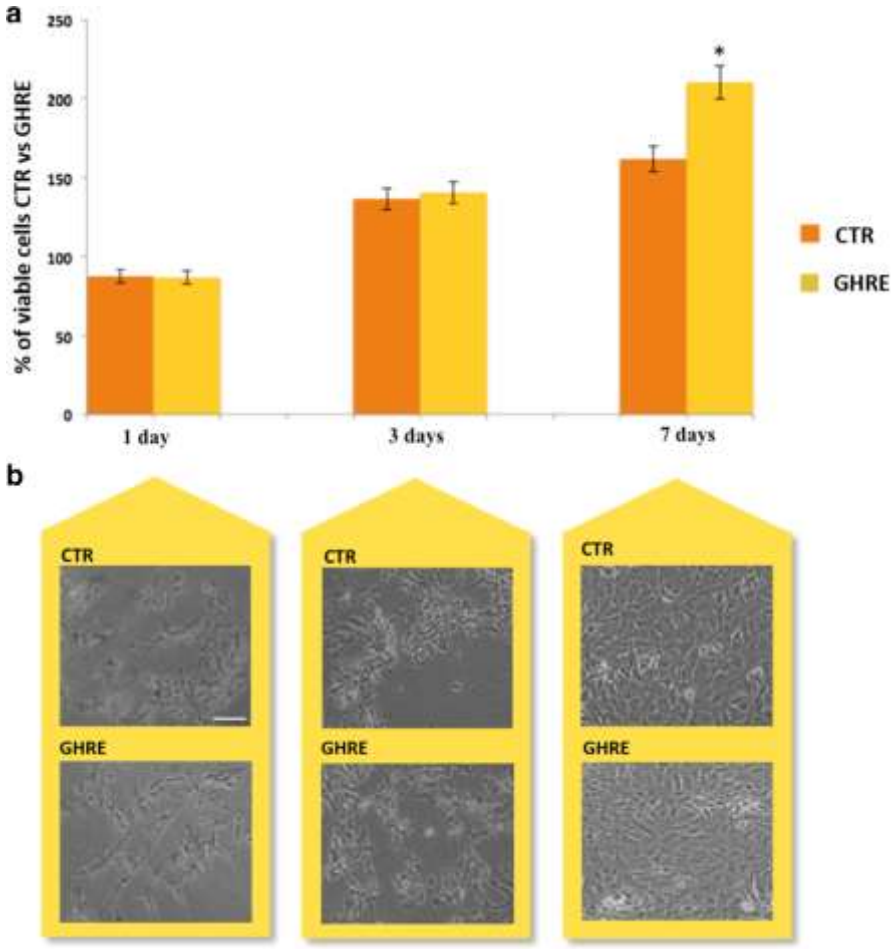

Fig. 1 Effect of Ghre on the viability of OECs during 7 days of treatment by using the MTT test. (a) The graph shows a statistically significant increase at the 7th day of treatment. $p < 0.05$ (*). (b) The figure shows representative OEC fields of qualitative analysis by phase contrast microscopy, cultured and treated for 1, 3, and 7 days. Scale bar: 50 μm

Figure 2

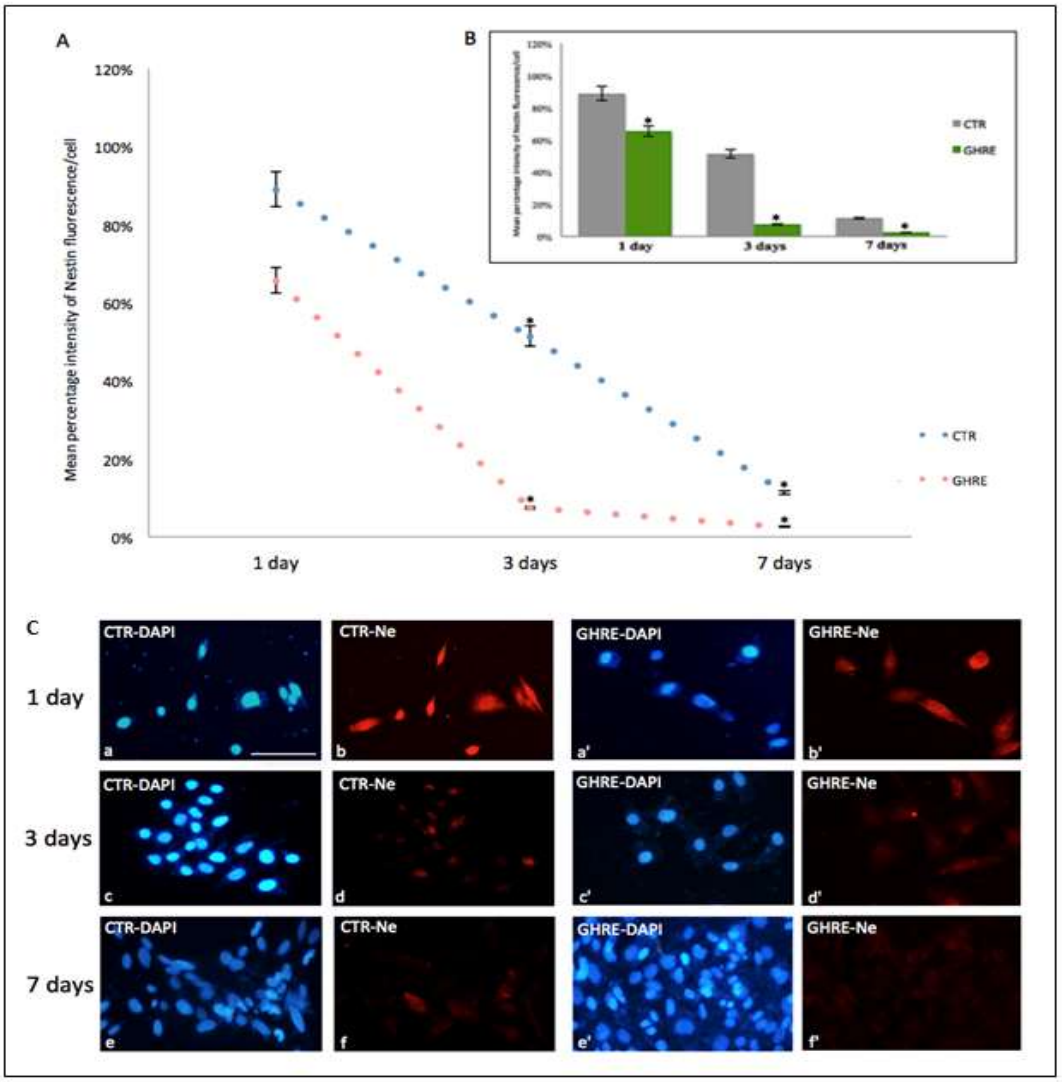

Fig. 2 Ne-positive OECs. (A) Different mean percentage intensities of Ne fluorescence/cell over 7 days in each experimental group (CTR and Ghre). (B) Comparison of mean percentage intensity of Ne fluorescence/cell between CTR and Ghre. (C) Representative images of both DAPI-labeled OECs (a, c, e, a′, c′, e′) and OEC-Ne immunoreactivity (b, d, f, b′, d′, f′). $p < 0.05$ (*). Scale bar: 50 μm

Figure 3

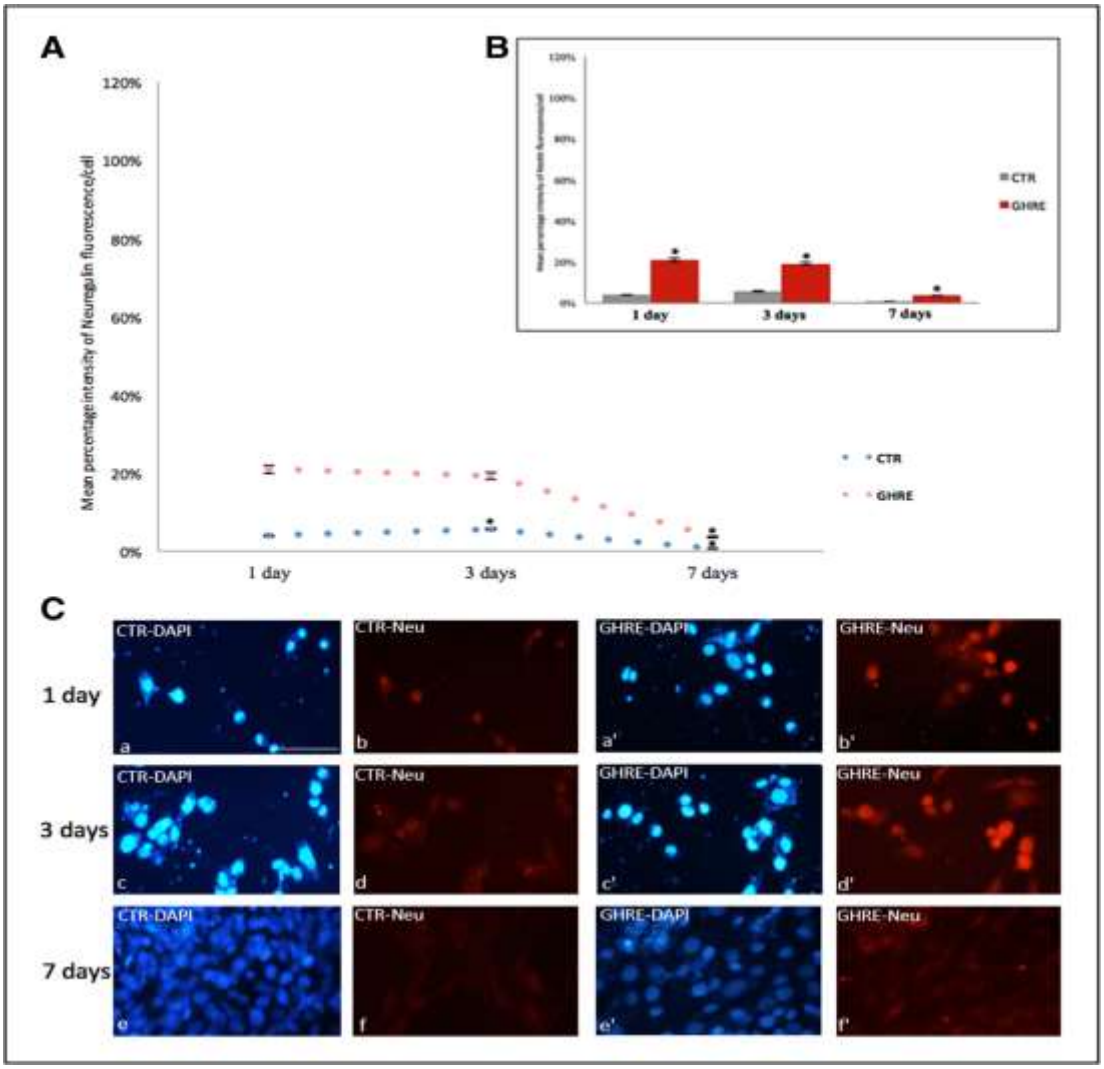

Fig. 3 Neu positive OECs. (A) Different mean percentage intensities of Neu fluorescence/cell over 7 days in each experimental group (CTR and Ghre). (B) Comparison of mean percentage intensity of Neu fluorescence/cell between CTR and Ghre. (C) Representative images of both DAPI-labeled OECs (a, c, e, a′, c′, e′) and OEC-Neu immunoreactivity (b, d, f, b′, d′, f ′). $p < 0.05$ (*). Scale bar: 50 μm

Figure 4

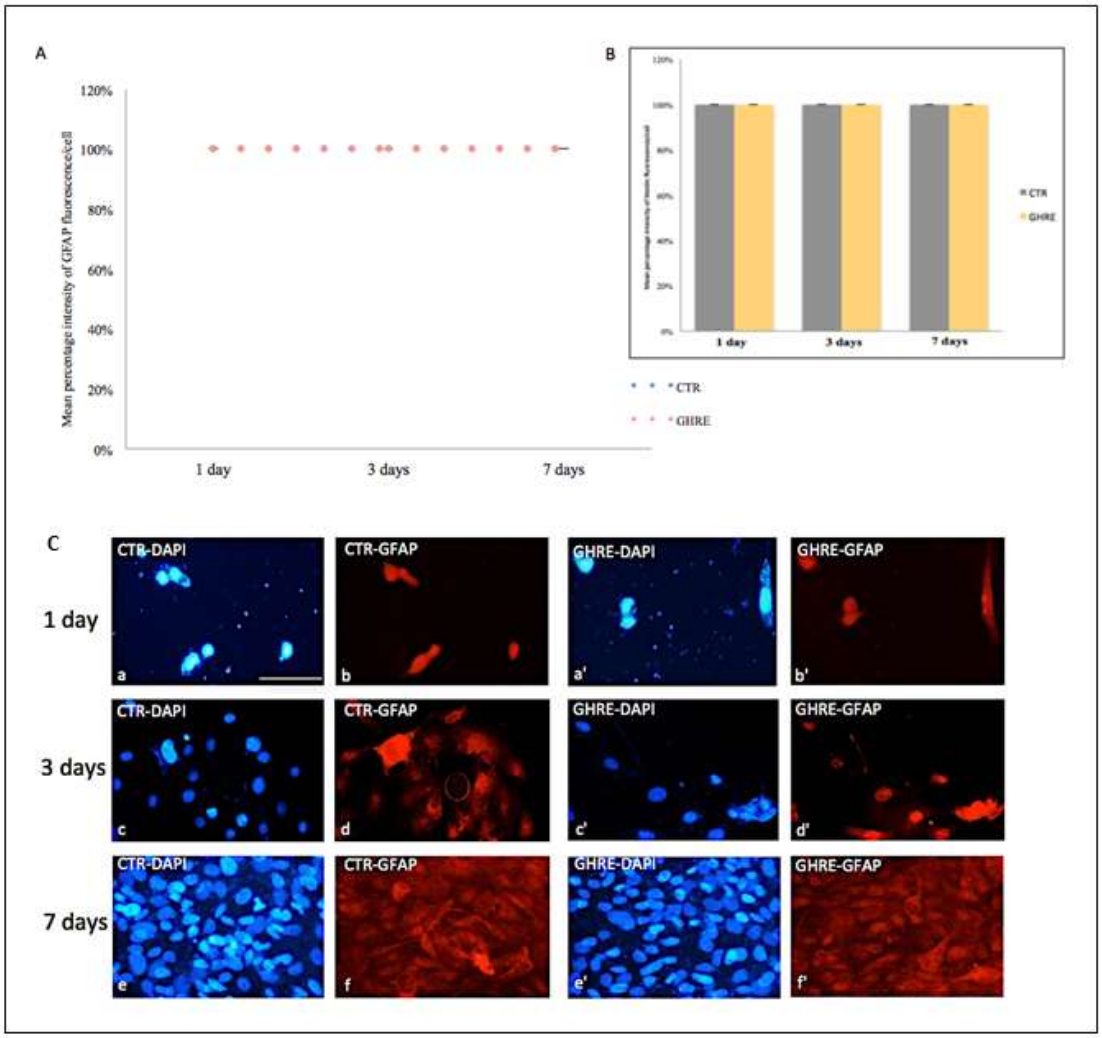

Fig. 4 GFAP-positive OECs. (A) Different mean percentage intensities of GFAP fluorescence/cell over 7 days in each experimental group (CTR and Ghre). (B) Comparison of mean percentage intensity of GFAP fluorescence/cell between CTR and Ghre. (C) Representative images of both DAPI-labeled OECs (a, c, e, a′, c′, e′) and OEC-GFAP immunoreactivity (b, d, f, b′, d′, f′). $p < 0.05$ (*). Scale bar: 50 μm

Figure 5

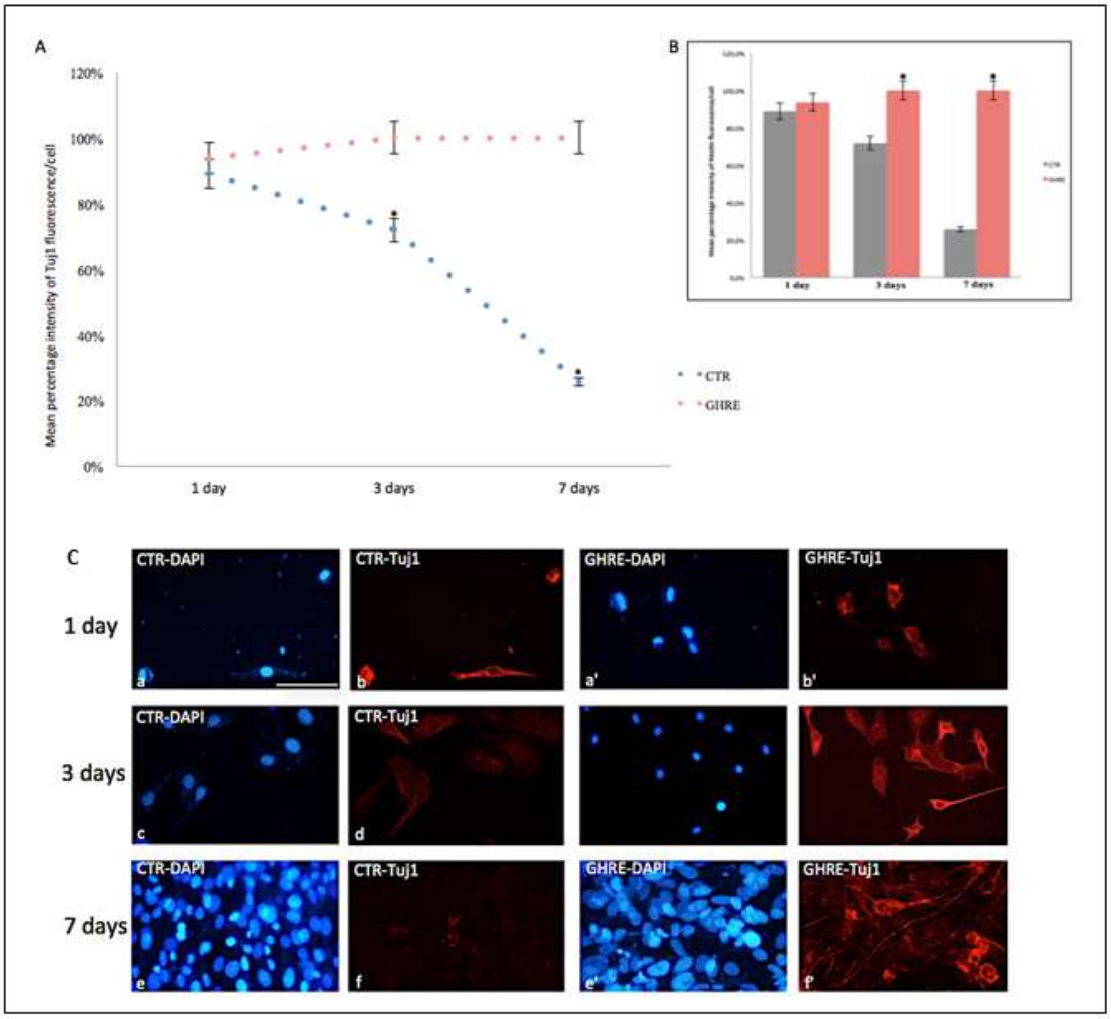

Fig. 5 Tuj1-positive OECs. (A) Different mean percentage intensities of Tuj1 fluorescence/cell over 7 days in each experimental group (CTR and Ghre). (B) Comparison of mean percentage intensity of Tuj1 fluorescence/cell between CTR and Ghre. (C) Representative images of both DAPI-labeled OECs (a, c, e, a′, c′, e′) and OEC-Tuj1 immunoreactivity (b, d, f, b′, d′, f′). $p < 0.05$ (*). Scale bar: 50 μm

Coculture of Axotomized Rat Retinal Ganglion Neurons with Olfactory Ensheathing Glia, as an In Vitro Model of Adult Axonal Regeneration.

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Abstract:

Olfactory ensheathing glia (OEG) cells are localized all the way from the olfactory mucosa to and into the olfactory nerve layer (ONL) of the olfactory bulb. Throughout adult life, they are key for axonal growing of newly generated olfactory neurons, from the lamina propria to the ONL. Due to their pro-regenerative properties, these cells have been used to foster axonal regeneration in spinal cord or optic nerve injury models.

We present an in vitro model to assay and measure OEG neuroregenerative capacity after neural injury. In this model, reversibly immortalized human OEG (ihOEG) is cultured as a monolayer, retinas are extracted from adult rats and retinal ganglion neurons (RGN) are

cocultured onto the OEG monolayer. After 96 h, axonal and somatodendritic markers in RGNs are analyzed by immunofluorescence and the number of RGNs with axon and the mean axonal length/neuron are quantified.

This protocol has the advantage over other in vitro assays that rely on embryonic or postnatal neurons, that it evaluates OEG neuroregenerative properties in adult tissue. Also, it is not only useful for assessing the neuroregenerative potential of ihOEG but can be extended to different sources of OEG or other glial cells.

Keyword

Olfactory Ensheathing Glia (OEG), Adult Axonal Regeneration, In Vitro Assay, Retinal Ganglion Neurons (RGN), Coculture, Axotomy

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INTRODUCTION

Adult central nervous system (CNS) neurons have limited regenerative capacity after injury or disease. A common strategy to promote CNS regeneration is transplantation, at the injury site, of cell types that induce axonal or neuronal growth such as stem cells, Schwann cells, astrocytes or olfactory ensheathing glia (OEG) cells [1,2,3,4,5].

OEG derives from the neural crest[6] and locates in the olfactory mucosa and in the olfactory bulb. In the adult, olfactory sensory neurons die regularly as the result of environmental exposure and they are replaced by newly differentiated neurons. OEG surrounds and guides these new olfactory axons to enter the olfactory bulb and to establish new synapses with their targets in the CNS[7]. Due to these physiological attributes, OEG has been used in models of CNS injury such as spinal cord or optic nerve injury and its neuroregenerative and neuroprotective properties become proven[8,9,10,11]. Several factors have been identified as responsible of the pro-regenerative characteristics of these cells, including extracellular matrix proteases production or secretion of neurotrophic and axonal growth factors[12,13,14].

Given the technical limitations to expand primary OEG cells, we previously established and characterized reversible immortalized human OEG (ihOEG) clonal lines, which provide an unlimited supply of homogeneous OEG. These ihOEG cells derive from primary cultures, prepared from olfactory bulbs obtained in autopsies. They were immortalized by transduction of the telomerase catalytic subunit (TERT) and the oncogene Bmi-1 and modified with the SV40 virus

large T antigen[15, 16, 17, 18]. Two of these ihOEG cell lines are Ts14, which maintains the regenerative capacity of the original cultures and Ts12, a low regenerative line that is used as a low regeneration control in these experiments[18].

To assess OEG capacity to foster axonal regeneration after neural injury, several in vitro models have been implemented.

In these models, OEG is applied to cultures of different neuronal origin and neurite formation and elongation— in response to glial coculture— are assayed. Examples of such neuronal sources are neonatal rat cortical neurons[19], scratch wounds performed on rat embryonic neurons from cortical tissue[20], rat retinal explants[21], rat hypothalamic or hippocampal postnatal neurons[22, 23], postnatal rat dorsal root ganglion neurons[24], postnatal mouse corticospinal tract neurons[25], human NT2 neurons[26], or postnatal cerebral cortical neurons on reactive astrocyte scar-like cultures[27]. In these models, however, the regeneration assay relies on embryonic or postnatal neurons, which have an intrinsic plasticity that is absent in injured adult neurons. To overcome this drawback, we present a model of adult axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs), based on the one originally developed by Wigley et al.[28, 29, 30, 31] and modified and used by our group[12, 13, 14, 15, 16, 17, 18, 32, 33]. Briefly, retinal tissue is extracted from adult rats and digested with papain.

Retinal cell suspension is then plated on either poly-lysine treated coverslips or onto Ts14 and Ts12 monolayers.

Cultures are maintained for 96 h before they are fixed and then immunofluorescence for axonal (MAP1B and NF-H proteins)[34] and somatodendritic (MAP2A and B)[35] markers is performed. Axonal regeneration is quantified as a percentage of neurons with axon, with respect to the total population of RGNs and axonal regeneration index is calculated as the mean axonal length per neuron. This protocol is not only useful for assessing the neuroregenerative potential of ihOEG but can be extended to different sources of OEG or other glial cells.

Protocol

NOTE: Animal experimentation was approved by national and institutional bioethics committees.

1. ihOEG (Ts12 and Ts14) culture

NOTE: This procedure is done under sterile conditions in a tissue culture biosafety cabinet.

1. Prepare 50 mL ME10 OEG culture medium as provided in Table 1.
2. Prepare 5 mL of DMEM/F12-FBS, as provided in Table 1, in a 15 mL conical tube.
3. Warm both media at 37 °C in a clean water bath, for 15 min.
4. Thaw Ts12 and Ts14 cells vials at 37 °C in a clean water bath.
5. Resuspend and add cells to the DMEM/F12-FBS culture medium prepared in step 2.

6. Centrifuge for 5 min at 300 x g.

7. Aspire the supernatant.

8. Add 500 μL of ME10 medium and resuspend the pellet.

9. Prepare a p60 cell culture dish with 3 mL of ME10 and add the cellular suspension, dropwise.

10. Move to distribute the cells uniformly across the plate.

11. Culture cells at 37 °C in 5% CO2.

NOTE: After reaching confluence, at least another passage must be done to optimize cells for coculture. 90% confluence is needed before seeding them on the coverslips for coculture. A confluent p-60 has a mean cell number of 7 x 105 for Ts14 and 2.5 x 106 for Ts12 cell lines. Ts12 and Ts14 cell lines should be passaged every 2–3 days.

2. Preparation of ihOEG (Ts12 and Ts14) for the assay

NOTE: This step must be done 24 h before RGN dissection and coculture.

1. Treat 12 mm Ø coverslips with 10 μg/mL poly-L-lysine (PLL) for 1 h. NOTE: The coverslips can be left overnight in PLL solution.

2. Wash the coverslips with 1x phosphate buffer saline (PBS), three times.

3. Detach Ts12 and Ts14 ihOEG cells from p60 cell culture dish.

1. Add 4 mL of DMEM/F12-FBS culture medium (Table 1) to a 15 mL conical tube. Warm at 37 °C in a clean water bath.

2. Remove the medium from plates and wash cells with 1 mL of 1x PBS-EDTA, once.

3. Add 1 mL of trypsin-EDTA to the OEG cells and incubate for 3–5 min at 37 °C, 5% CO2.

4. Collect cells with a p1000 pipette and transfer them to medium prepared in step 3.1.

5. Centrifuge for 5 min at 200 x g.

6. Aspire the supernatant.

7. Add 1 mL of ME10 medium and resuspend the pellet.

8. Count the cell number in a hemocytometer.

4. Seed 80,000 Ts14 cells or 100,000 Ts12 cells onto the coverslips in 24-well plates in 500 µL of ME10 medium.

5. Culture cells at 37 °C in 5% CO2, for 24 h.

3. Retinal tissue dissection

NOTE: 2-month old male Wistar rats are used as RGN source. Two retinas (one rat) for 20 wells of a 24-well cell dish. Autoclave surgical material before use. Papain dissociation kit is commercially purchased (Table of Materials). Follow the provider´s instructions for reconstitution. Reconstitute D,L-2-amino-5-phosphonovaleric acid (APV) in 5 mM stock and prepare the aliquots.

1. On the day of the assay, prepare the following media.

1. Prepare a p60 cell culture dish with 5 mL of cold EBSS (vial 1 of the papain dissociation kit).

2. Prepare a p60 cell culture dish with reconstituted vial 2 (papain) of the papain dissociation kit plus 50 µL of APV, 250 µL of vial 3 (DNase) plus 5 µL of APV.
3. In a sterile tube mix 2.7 mL of vial 1 with 300 µL of vial 4 (albumin-ovomucoid protease inhibitor). Add 150 µL of vial 3 (DNase) plus 30 µL of APV.
4. Prepare 20 mL of Neurobasal-B27 medium (NB-B27) as provided in Table 1.

2. Sacrifice a rat by asphyxiation with CO2.

3. Remove the head by decapitation with guillotine; place it in a 100 mm Petri dish and spray the head with ethanol 70% before placing it in a laminar flow hood.

4. Cut the rat´s whiskers with scissors so they do not interfere with the eye manipulation.

5. Grip the optic nerve with forceps to pull out the eyeball enough to be able to make an incision across the eye with a scalpel.

6. Remove the lens and vitreous humor and pull out the retina (orange-like tissue), while the remaining layers of the eye stay inside (including the pigment epithelial layer).

7. Place the retina in the p60 cell culture dish prepared in step 3.1.1.

8. Transfer the retina to the p60 cell culture dish prepared in step 3.1.2 and cut it with the scalpel in small pieces of an approximate size < 1 mm.

9. Transfer to a 15 mL plastic tube. 10. Incubate the tissue for 30 min, in a humidified incubator at 37 °C under 5% CO2, with agitation every 10 min11. Dissociate cell clumps by pipetting up and down with a glass Pasteur pipette.

12. Centrifuge the cell suspension at 200 x g for 5 min.

13. Discard supernatant and to inactivate papain, resuspend the cell pellet in the solution prepared in step 3.1.3. (1.5 mL for 2 eyes).

14. Carefully pipet this cell suspension into 5 mL of reconstituted vial 4.

15. Centrifuge at 200 x g for 5 min.

16. While centrifuging, completely remove the ME-10 medium from the OEG 24 well cell plate (previously prepared in step 2) and replace it with 500 μL of NB-B27 medium per well.

17. Discard the supernatant and resuspend the cells in 2 mL of NB-B27 medium.

18. Plate 100 μL of retinal cell suspension, per well of the m24 plate, onto PLL-treated or OEG monolayers-coverslips.

19. Maintain cultures at 37 °C with 5% CO2 for 96 h in NBB27 medium.

4. Immunostaining

1. After 96 h, fix the cells for 10 min by adding the same volume of 4% paraformaldehyde (PFA) in 1x PBS to the culture medium (600 μL) (PFA final concentration 2%).
2. Remove the media and PFA from the 24-multiwell plate and once again add 500 μL of 4% paraformaldehyde (PFA) in 1x PBS. Incubate for 10 min.
3. Discard the fixer and wash 3 times with 1x PBS for 5 min.
4. Block with 0.1% Triton X-100/1% FBS in PBS (PBS-TS) for 30–40 min. 5. Prepare the primary antibodies in PBS-TS buffer as follows: SMI31 (against MAP1B and NF-H proteins) monoclonal antibody (1:500). 514 (recognizes MAP2A and B proteins) rabbit polyclonal antiserum (1:400).
6. Add primary antibodies to cocultures and incubate overnight at 4 °C.
7. Next day, discard the antibodies and wash the coverslips with 1x PBS, 3 times, for 5 min.

8. Prepare the secondary antibodies in PBS-TS buffer as follows: For SMI-31, anti-mouse Alexa Fluor 488 (1:500). For 514, anti-rabbit Alexa-594 (1:500).
9. Incubate cells with the corresponding fluorescent secondary antibodies for 1 h, at RT, in the dark.
10. Wash the coverslips with 1x PBS, 3 times, for 5 min, in the dark.
11. Finally, mount coverslips with mounting medium (Table of Materials) and keep at 4 °C.

NOTE: Whenever necessary, fluorescent nuclei staining with DAPI (4,6-diamidino-2-phenylindole) may be performed. Before mounting, incubate the cells for 10 min in the dark with DAPI (10 μg/mL in 1x PBS). Wash the coverslips 3 times with 1x PBS and finally, mount the coverslips with the mounting medium.

5. Axonal regeneration quantification

NOTE: Samples are quantified under the 40x objective of an epifluorescence microscope. A minimum of 30 pictures should be taken on random fields, with at least 200 neurons, to be quantified for each treatment. Each experiment should be repeated a minimum of three times.

1. Quantify the percentage of neurons with axon (SMI31 positive neurite) relative to the total population of RGNs(identified with

MAP2A/B 514 positive immunostaining of neuronal body and dendrites).

2. Quantify the axonal regeneration index or mean axonal length (μm/neuron). This parameter is defined as the sum of the lengths (in μm) of all identified axons, divided by the total number of counted neurons, whether they presented an axon or not. Axonal length is determined using the plugin NeuronJ of the image software ImageJ (NIH-USA).

3. Calculate the mean, standard deviation, and statistical significance using the appropriate software.

Representative Results

In this protocol, we present an in vitro model to assay OEG neuroregenerative capacity after neuronal injury. As shown in Figure 1, the OEG source is a reversible immortalized human OEG clonal cell line -Ts14 and Ts12-, which derives from primary cultures, prepared from olfactory bulbs obtained in autopsies[15 , 17 , 18] . Retinal tissue is extracted from adult rats, digested, and retinal ganglion neurons (RGN) suspension is plated on either PLL-treated coverslips or onto ihOEG monolayers, Ts14 or Ts12. Cultures are maintained for 96 h before they are fixed. Axonal and somatodendritic markers are analyzed by immunofluorescence and axonal regeneration is quantified.

Ts14 OEG identity is assessed by immunostaining with markers described to be expressed in ensheathing glia (Figure 2), such as S100 β (2A) and vimentin (2B); GFAP expression was also analyzed to discard astrocyte contamination (2C). As shown, Ts14 expressed S100 β and vimentin but not GFAP.

In the axonal regeneration assay, Ts14 regenerative capacity is compared to Ts12 in RGN-OEG cocultures, using PLL substrate as a negative control (Figure 3). Both the percentage of cells with axons as well as the average length of the regenerated axons were significantly higher in neurons cocultured on Ts14 monolayers, compared to neurons plated on either Ts12 cells or PLL (Figure 3D,E). Representative images show a lack of capacity of RGN to regenerate their axons over PLL or Ts12 cells (Figure 3A,B), while Ts14 stimulates the outgrowth of axons in RGN (3C).

Discussion

OEG transplantation at CNS injury sites is considered a promising therapy for CNS injury due to its constitutive pro-neuroregenerative properties[7, 8, 9]. However, depending on the tissue source—olfactory mucosa (OM-OEG) versus olfactory bulb (OB-OEG)—or the age of the donor, considerable variation exists in such capacity[26, 31, 33, 36].

Therefore, it is of importance to have an easy and reproducible in vitro model to assay the neuroregenerative capacity of a given OEG sample, before initiating in vivo studies. In this protocol, adult rats' axotomized RGN are cocultured onto a monolayer of the OEG to assay.

Subsequent analysis of RGN axonal and somatodendritic markers by immunofluorescence is performed to assess RGN axonal regeneration.
An initial difficulty of the assay is the source of OEG. In this work, we use reversible immortalized human OEG (ihOEG) clonal lines, previously established and characterized by our group[15, 16, 17, 18,] which provide an unlimited supply of homogeneous OEG. Two of these ihOEG cell lines are Ts14, which maintains the regenerative capacity of the original cultures and Ts12, a low regenerative line that is used as a low regeneration control in these experiments[18]. Nevertheless, although technical limitations exist to expand human primary OEG cells, they can also be obtained from nasal endoscopic biopsies—OM—or, in case of OB-OEG, from cadaver donors.

Preparation of monolayer OEG cultures is a crucial procedure, as too many cells could cause the coculture to detach from the plate. Therefore, prior to OEG preparation for the assay, it is recommended that the user determines the optimal number of cells to be plated, depending on their size and division rate.
Another critical issue is the retinal tissue dissociation, after retina dissection. It is necessary to break up the tissue fragments, following incubation in the dissociation mix. If done too vigorously, the cells will be destroyed, but tissue fragments will be left intact if done too weakly. In order to obtain a homogeneous cell suspension, we suggest filling

and emptying a Pasteur pipette 10–15 times, with a tip of intermediate diameter, while avoiding bubbling. Pasteur pipettes with wide tips can be narrowed using a Bunsen burner.

To assess the capacity of different glial populations to foster adult neurons' axonal regeneration, we have determined that 96 h is the time interval that best suits the aim because: 1) it is the longest time to maintain the culture alive without disturbing the OEG monolayer; and 2) it is the time needed for neurons to grow axons long enough to reveal differences between the regenerative capacities of different OEG populations or other non-regenerative cells (i.e., fibroblasts[12, 13, 14, 15, 16, 17, 18, 32, 33]. It would certainly be interesting to determine the time course of the regeneration process, as it could provide information about the differential regenerative properties of different glial populations, at shorter times of the co-culture. In our hands, for regenerative glia, the time course between 72–96 h is quite similar for all the cell lines, although axons are shorter at 72 h (unpublished data). Also, 96 h of co-culture, permits to study OEG-dependent mechanisms of adult axonal regeneration[12, 14].

During axonal regeneration quantification, it is important to take a minimum of 30 pictures at 400 augments (40x objective), at different random areas of the coverslip, but following the complete axons of the photographed neurons. Therefore, the experimenter must take serial pictures in the chosen areas to measure the real axonal lengths.

Other in vitro approaches have also been developed to evaluate OEG regenerative functions. In these models, OEG is applied to cultures of

different neuronal origin and, in response to glial coculture, neurite formation and elongation are assayed[19,20,21,22,23,24,25,26,27]. However, the regeneration assay relies on embryonic or postnatal neurons, which have an intrinsic plasticity absent from injured adult neurons. This model consisting of adult axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs) overcomes this drawback. In addition, we are dissecting adult retinas, and because we cut optic nerve and axons retract in the process of dissection, we obtain neuronal bodies clean of myelin, to perform the coculture. This is the difference with other parts of the adult CNS, where myelin can hinder very much with the dissection to obtain clean neurons for the coculture. Based on the one originally developed by Wigley et al.[28,29,30,31], we highlight the following improvements in the protocol. First, the use of neurobasal medium supplemented with B27 as OEG-RGN coculture medium, which allows growth of neuronal cells and positively affects the reproducibility of the experiment. Second, we characterize and quantify axonal regeneration by using a specific marker of the axonal compartment; and third, we use an additional direct parameter, the mean axonal length/neuron, that assesses the axonal growth regenerative potential of OEG.

In summary, we consider that this is a simple, reproducible, time saving, and medium-cost assay, not only useful for assessing the neuroregenerative potential of ihOEG, but also because it can be extended to different sources of OEG or other glial cells. Moreover, it could be used as a valuable proof of concept of the neuroregenerative

potential of an OEG or glial sample, before translation to in vivo or clinical studies.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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Figure 1

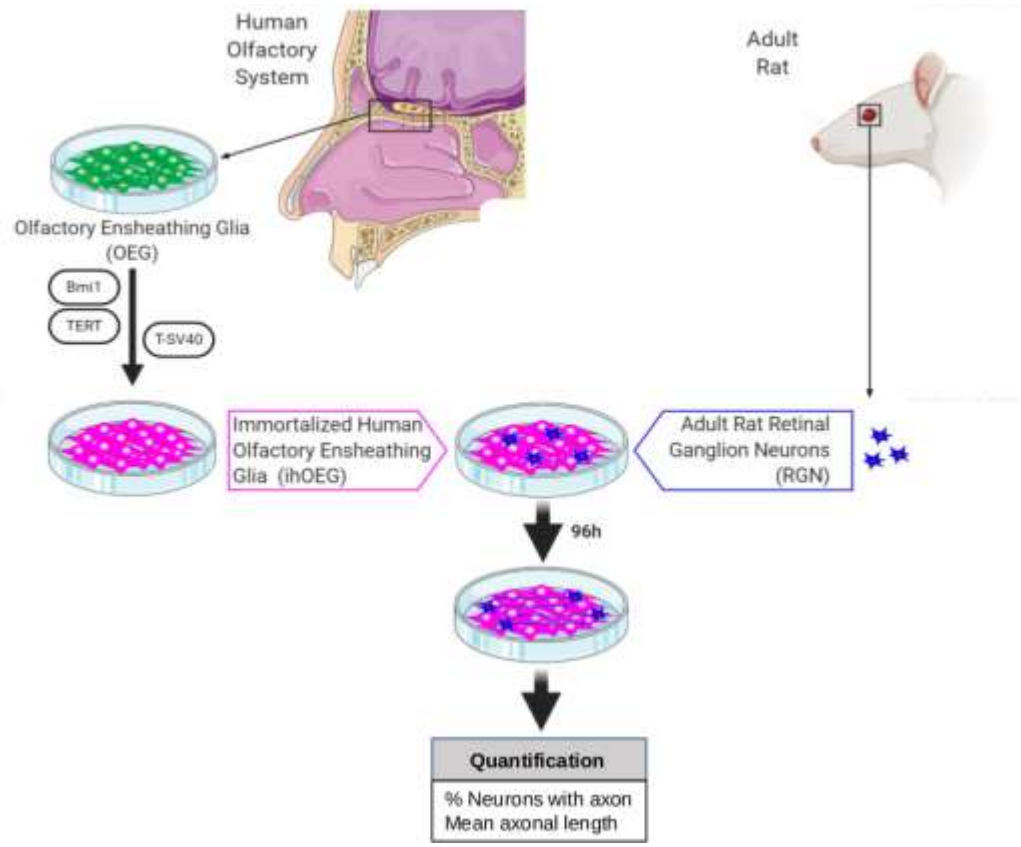

Figure 1: Diagram of rat retinal ganglion neurons with olfactory ensheathing glia cells coculture, as a model of adult axonal regeneration. Immortalized human OEG (ihOEG) clonal cell lines -Ts12 and Ts14- derived from primary cultures from olfactory bulbs. Retinal ganglion neurons from adult rats are plated on either PLL-treated coverslips (negative control) or onto Ts14 or Ts12 monolayers. Cultures are maintained for 96 h before they are fixed and axonal and somatodendritic markers are analyzed by immunofluorescence. Percentage of neurons with axon and mean axonal length/neuron are quantified to assay RGN axonal regeneration.

Figure 2

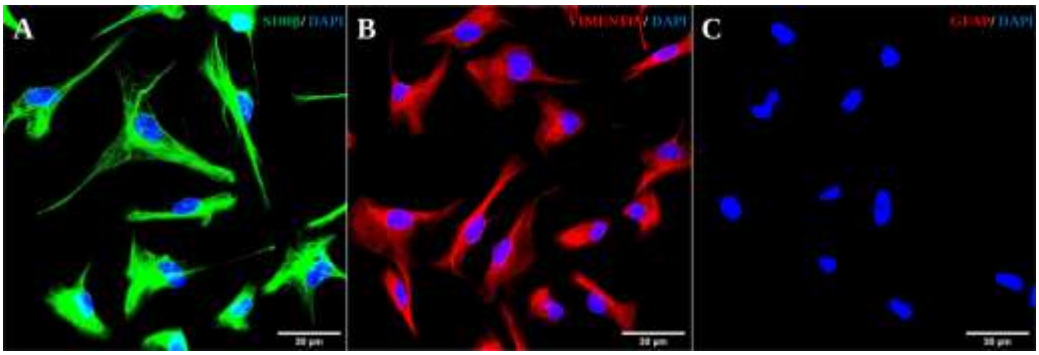

Figure 2: Identity of ihOEG cell line Ts14. Immunofluorescence images of Ts14 in culture, labeled with anti-S100 β (panel A, green) and vimentin (panel B, red). GFAP expression (panel C, red) was also analyzed to discard astrocyte contamination. Nuclei are stained with DAPI (blue).

Figure 3

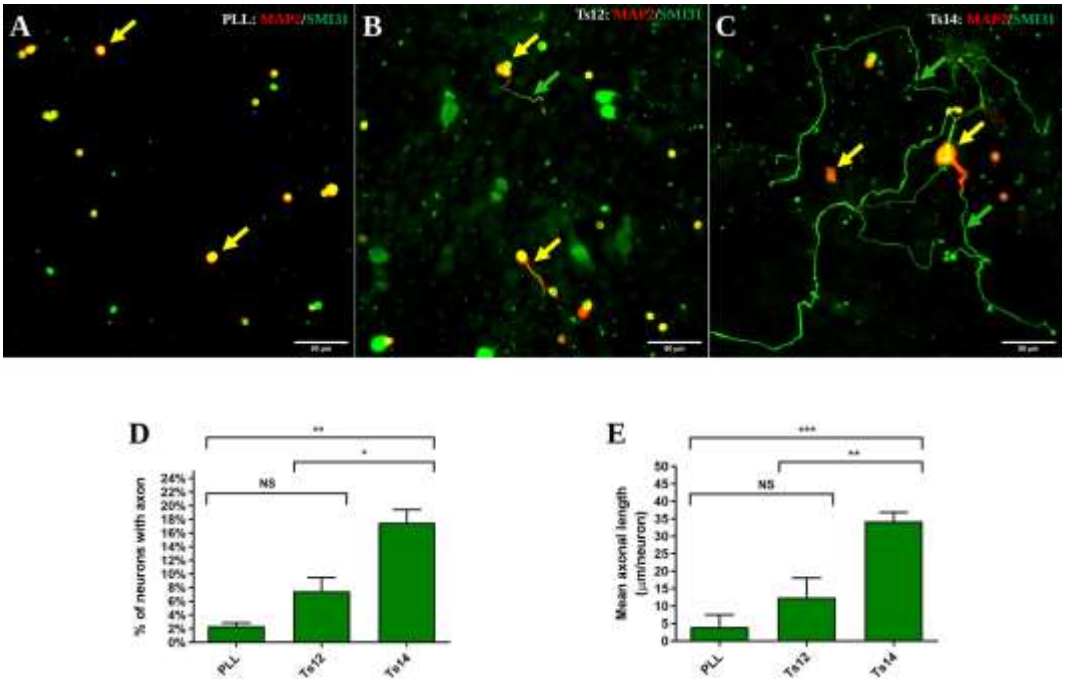

Figure 3: Assay for axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs). (A–C) Immunofluorescence images showing somatodendritic labelling with 514 antibodies, which recognizes microtubule-associated protein MAP2A and B, in red, and with axon-specific SMI31 antibody in green, against MAP1B and NF-H proteins. Green arrows indicate RGN axons (SMI31-positive: green) and yellow arrows indicate neuronal bodies and dendrites (514 positive: red and yellow). (D,E) Graphs show mean and standard deviation of the percentage of neurons exhibiting axons and the axonal regeneration index, a parameter reflecting the mean axonal length (µM) of axons per neuron. A minimum of 30 pictures (40x) were taken on random fields and quantified for each cell sample. Experiments were performed in triplicate, from three different rats (N = 3), retinal tissue pooled from both eyes, with duplicates for each experimental condition (each glia population tested). Asterisks indicate the statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS: non significance (ANOVA and post hoc Tukey test comparisons between parameters quantified for Ts14 vs Ts12, Ts14 vs PLL, and Ts12 vs PLL).

CONCLUDING REMARKS

In the last decade, several researches have shown that Ghre has regulatory roles in many organs and systems (Kojima et al. 1999; Lu et al. 2002; Carlini et al. 2004; Ferrini et al. 2009; Russo et al., 2017). The binding between Ghre and its receptor, GHS-R 1a, determines the activation of several intracellular signalling pathways. In particular, it induces the increase of the inositol phosphate cascade and the activation of protein kinase C, which in turn activates the release of intracellular calcium and also, the phosphorylation of ERK1/2, the induction of PI-3 K and Akt phosphorylation (Mousseaux et al., 2006).

Ghre has recently been associated with several diseases, such as Alzheimer, through its neuroprotective and anti-apoptotic functions (Cecarini et al., 2016; Collden et al., 2017; Seminara et al., 2018).

Recent research has focused on the role of Ghre in the olfactory processes (Tong et al. 2011).

Ghre is involved in cognitive mechanisms and eating behaviour, and two regions of the brain are mostly involved in the regulation of feeding behaviour, the amygdala and the hypothalamus. Although direct connections from the mitral cells (MCs) of the OB to the amygdala have been reported (Pro-Sistiaga et al 2007), there is still no evidence that the same occurs from the hypothalamus, although, this area can be reached indirectly through the amygdala (Saper et al. 2002; Kang et al. 2011; Trellakis et al. 2011).

In our neuroanatomical study, we shown that Ghre axonal fibres from the MCs, can transmit olfactory information via branching connections

to the amygdala and hypothalamus. This study highlights the central effects of Ghre that, at the mitral cell level of the OB, could modulate the response to smell.

Furthermore, it is also reported that, the ability to search food through the sense of smell seems to be improved by the presence of GHS-R 1a in the olfactory circuits (Tong et al., 2011). In fact, the Ghre produced by the Mitral Cells (MC), interacts with the GHS-R 1a expressed on the OEC membranes. It is known that Ghre promotes the axonal growth and the synaptogenesis of neurons, acting like a neurotrophic factor (Stoyanova et al., 2014), and moreover that the OECs are a source of trophic factors which allow them interfacing with their environment (Pellitteri et al., 2010; Khankan et al., 2015). Therefore, the binding between Ghre and GHSR 1a could stimulate the OECs to release Ghre which, in turn, would promote the axonal elongation of the ORN, which are in interaction with the OEC receptors through the release of glutamate and ATP (Lohr et al., 2014).

OECs through Ghre can improve the growth of neurites and promote their alignment; in addition, as these cells exert neuroprotective role, the Ghre expressed by the OECs could contribute to the synaptic interaction of ORN and MC. Several studies considered the OECs as leading reparative candidate, when transplanted into the injured spinal cord, they are able to merge with astrocytes and to migrate (Lakatos et al., 2000) to areas distal from the transplantation site, forming the gap between the lesion site and normal spinal cord compared with other cells (Li et al., 2012).

Therefore, as expressed above, the OECs through the Ghre release, can interact like a reinforcing function, in the peripheral olfactory circuit, providing neurotrophic support to the synaptic interaction between olfactory neurons and MC. Ghre, through the binding with its receptor, could modulate the expression of biomarkers acting in a paracrine and autocrine.

Moreover, being known that Ghre has a neurotrophic action, both in promoting axonal growth and synaptogenesis, it could be hypothesized that even the OECs through the Ghre, can improve the growth of neurites and promote alignment. Therefore, it is possible to consider this peptide as potential support for the OECs in the cellular therapy in different neurological disorders and injured CNS.

It might be desirable that the results obtained could be applied in vivo on animal models of neurodegenerative diseases.

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