Ghrelin: A novel neuromuscular recovery promoting factor?

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ABSTRACT

Promoting neuromuscular recovery after neural injury is a major clinical issue. While techniques for nerve reconstruction are continuously improving and most peripheral nerve lesions can be repaired today, recovery of the lost function is usually unsatisfactory. This evidence, claims for innovative non-surgical therapeutic strategies that can implement the outcome after neural repair.

Although no pharmacological approach for improving posttraumatic neuromuscular recovery has still entered the clinical practice, various molecules are explored in experimental models of neural repair. One of such molecule is the circulating peptide hormone ghrelin. This hormone has proven to have a positive effect on neural repair after central nervous system lesion and very recently his effectiveness has also been demonstrated in preventing posttraumatic skeletal muscle atrophy. By contrast, no information is still available about its effectiveness on peripheral nerve regeneration although preliminary data from our laboratory suggest that this molecule can have an effect also in promoting axonal regeneration after nerve injury and repair.

Should this been confirmed, ghrelin might represent an ideal candidate as a therapeutic agent for improving posttraumatic neuromuscular recovery because of its putative effects at all the various structural levels involved in this regeneration process, namely, the central nervous system, the peripheral nerve, and the target skeletal muscle.

1. Introduction

One of the most frequent causes of movement impairment are lesions of peripheral nerves that induce dramatic muscle atrophy and can occur as a consequence of a variety of traumas (e.g. work accidents) and diseases (e.g. diabetes) with high social costs (de Putter et al., 2012).

The consequences of nerves injuries may be disastrous and can result in substantial functional loss. The increasing number of patients receiving nerve surgery represents an enormous stimulus for more research in peripheral nerve regeneration and, most of all, for defining innovative strategies for improving functional recovery of repaired nerves.

The peripheral nerve regeneration is usually far from satisfactory (Navarro, Vivo, & Valero-Cabre, 2007; W. Sun et al., 2009) and there is no technique to guarantee total recovery and normalization of functional sensibility following repair of an injured nerve.

The significant improvements made in understanding the basic biological and molecular mechanisms underlying the progression of nerve regeneration have resulted in the identification of a number of key molecules involved in the process. Among these molecules there are environmental factors (laminin, integrin, dystroglycan, L-periaxin, fibrin), neurotrophic factors (such as glial-cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), Neuregulin1 (NRG1), Transforming growth factor-β (TGF β)), cytokines (interleukin-6 (IL-6), leukemia inhibitory factor (LIF)), transcription factors (signal transducer and activator of transcription 3 (STAT3)), plasmalemma-associated PKC substrates (GAP-43, myristoylated alanine-rich c kinase substrate (MARCKS), and cytoskeleton-associated protein (CAP) 23) (Chen, Yu, & Strickland, 2007).

A number of hormones have also been shown to play a regenerative-promoting role after a peripheral nerve injury. Progesterone has been demonstrated to be involved in remyelination: blocking its action, the thickness of myelin sheaths of remyelinated axons after injury decrease. In contrast, administration of either exogenous progesterone or its precursor pregnenolone to the injured site promotes myelin sheath formation (Koenig et al., 1995). Also thyroid hormone (T3) plays an important role in neuronal maturation and myelination. Administration of T3 to an injured rat sciatic nerve results in an increased number of remyelinated fibers, along with the increment of their diameter and myelin thickness compared with control nerves (Mercier, Turque, & Schumacher, 2001). Parathyroid hormone–related peptide (PTHrP) is widely expressed in the PNS and is upregulated in Schwann cells following sciatic nerve crush (Macica, Liang, Lankford, & Broadus, 2006). Addition of PTHrP to dorsal root ganglion explants stimulates Schwann cell

migration, but does not affect their proliferation and survival (Macica et al. 2006). After sciatic nerve crush, the expressions of erythropoietin (Epo) and Epo receptors (EpoR) are increased in Schwann cells. Addition of exogenous Epo to injured sciatic nerves stimulates Schwann cell proliferation (X. Li, Gonias, & Campana, 2005) and reduces the expression of tumor necrosis factor alpha (TNF-α) in Schwann cells at the injured site, improving peripheral nerve regeneration (Campana et al., 2006). Growth hormone (GH)-treated rats showed improved functional recovery and axonal remyelination after rat sciatic nerve injury (Devesa et al., 2012).

Alpha-melanocyte stimulating hormone (alphaMSH) and corticotropin (ACTH) are known to improve the post-lesion repair of injured peripheral nerves by accelerating and enhancing nerve regeneration and muscle reinnervation (Strand et al., 1993). Moreover, alphaMSH has been shown to stimulate the sprouting and neuritogenesis from spinal and sensory neurons in vitro (van der Neut, Hol, Gispen, & Bar, 1992). In addition, stimulation with alphaMSH of spinal cord trauma showed a profound and significant stimulation of neurite outgrowth (Joosten, Majewska, Houweling, Bar, & Gispen, 1999).

The ability of gonadal steroids to enhance functional recovery and outgrowth rates after nerve injury was also demonstrated. Indeed, rats subjected to crush injury of the sciatic nerve and treated with testosterone propionate showed a faster regeneration of axonal regrowth. Accelerating regeneration rate was also obtained after exposure with the non-aromatizable androgen, dihydrotestosterone and E2 (Jones, Alexander, Brown, & Tanzer, 2000).

2. Ghrelin: an overview on its role in physiological and pathological conditions

Ghrelin is a circulating peptide hormone, acylated on Ser 3, that, through binding and activation of its receptor GHSR-1a in hypothalamus and pituitary, promotes a potent release of GH (Kojima et al., 1999). Ghrelin is now widely acknowledged as the “hunger hormone”, because, alongside the GH-releasing effect, its secretion, regulated by fasting, stimulates food intake, promotes adiposity, and controls energy homeostasis, (Tschop, Smiley, & Heiman, 2000; Wren et al., 2001).

Plasma ghrelin levels, normally high during fasting and falling to basal values after the assumption of food, are altered in pathological conditions affecting body mass and/or body energy metabolism. Circulating ghrelin concentrations negatively correlate with body mass index (BMI) and are lower in overweight or obese subjects compared with normal subject (Tschop et al., 2001). On the other hand, plasma ghrelin levels increase in conditions characterized by energy deficiency such as anorexia nervosa (Ariyasu et al., 2001) or anorexia/cachexia associated to cancer (Garcia et

al., 2005; Shimizu et al., 2003), chronic heart failure (Nagaya et al., 2001), chronic kidney disease (Yoshimoto et al., 2002), and chronic obstructive pulmonary disease (Itoh et al., 2004).

Beside the regulation of feeding, ghrelin exhibits also a broad array of other biological activities on the cardiovascular systems, where it decreases blood pressure (Lambert et al., 2011) and improves cardiac function after heart damage (Nagaya et al., 2004; Nagaya et al., 2001). Moreover, it inhibits the apoptosis of cardiomyocytes and endothelial cells by activating PI3K/Akt and ERK-1/2 pathways *in vitro* (Baldanzi et al., 2002). Ghrelin has also an anti-inflammatory action, inhibiting the activity of the transcription factor NF-kB, and suppressing the production of proinflammatory cytokines (Balasubramaniam et al., 2009; Dixit et al., 2004; Lee, Kim, Li, & Park, 2012; W. G. Li et al., 2004).

Acylation is essential for ghrelin activity through GHSR-1a: the most abundant unacylated circulating form of ghrelin does not bind to and activate GHSR-1a (Porporato et al., 2013). Far from being the inactive byproduct of acylated ghrelin metabolism, a constantly increasing number of evidences proves that unacylated ghrelin is a biologically active peptide, sharing most of acylated ghrelin peripheral activities and participating in the regulation of food intake and adipogenesis through mechanisms not fully elucidated, but very likely independent from GHSR-1a (Asakawa et al., 2005; Toshinai et al., 2006).

Among the wide range of common biological functions, acylated and unacylated ghrelin exert a protective activity on several cell lines including cardiac and endothelial cells, β-pancreatic cells, human pancreatic islets, and cortical neurons (Baldanzi et al., 2002; Chung, Seo, Moon, & Park, 2008; Granata et al., 2007). Moreover, both peptides promote differentiation of skeletal myoblasts, Leydig cells, preadipocytes, and human cardiac embryonic stem cells (Barreiro et al., 2004; Filigheddu et al., 2007; Gao et al., 2012; Liu, Chen, Xu, Vicaut, & Sercombe, 2009).

The findings that unacylated ghrelin shares with ghrelin common binding sites in cell lines, including cells lacking GHSR-1a (Baldanzi et al., 2002; Cassoni et al., 2001; Filigheddu et al., 2007; Granata et al., 2007; Jeffery, Herington, & Chopin, 2002) and that both peptides have common biological activities in vivo, also in *Ghsr* null mice (Porporato et al., 2013), strongly suggest that both peptides act through a common, although yet unidentified, receptor.

In both human patients and experimental models ghrelin administration ameliorates the cachectic state associated to several pathological conditions such as chronic heart failure (Nagaya et al., 2004), chronic kidney disease (Deboer et al., 2008), arthritis, cancer (Argiles & Stemmler, 2013; DeBoer et al., 2007; Neary et al., 2004), burn injuries (Balasubramaniam et al., 2006), and COPD (Nagaya et al., 2005).

Ghrelin treatment for cancer cachexia is a good candidate for muscle wasting treatment because

ghrelin levels are elevated in cancer cachexia – suggesting a compensatory effect – and it controls mediators involved in the cachectic process (Argiles & Stemmler, 2013).

Since ghrelin induces GH release – and therefore the activation of GH/IGF-1 axis –, promotes food intake, exhibits anti-inflammatory activities, and stimulates positive energy balance, it has been obviously assumed that the beneficial effect of ghrelin on the cachectic state was a consequence of its activity mediated by GHSR-1a. Although ghrelin may undeniably inhibit cachexia through these GHSR-1a-mediated activities, several evidences prove that both acylated and unacylated ghrelin have a direct anti-atrophic activity in skeletal muscles: unacylated ghrelin, which does not bind GHSR-1a and does not activate the GH/IGF-1 axis, reduces burn-induced skeletal muscle proteolysis and local TNF-α upregulation in rats (Sheriff et al., 2012); the cardiac over-expression of the ghrelin gene, resulting in the upregulation of circulating unacylated ghrelin, counteracts muscle atrophy induced by either fasting or denervation; and, finally, both acylated and unacylated peptides impairs fasting-induced atrophy in *Ghsr* null mice (Porporato et al., 2013).

Ghrelin anti-atrophy effect in muscle was also demonstrated using a mouse model of hindlimb suspension (HS), where ghrelin administration was shown to diminish the reduction of hindlimb muscle mass, thus facilitating the recovery from muscle atrophy (Koshinaka et al., 2011).

3. Ghrelin and the nervous system

Ghrelin receptor GHSR-1a is detected in various hypothalamic nuclei (anteroventral preoptic nucleus, anterior hypothalamic area, suprachiasmatic nucleus, lateroanterior hypothalamic nucleus, supraoptic nucleus, ventromedial hypothalamic nucleus, arcuate nucleus, paraventricular nucleus, and tuberomammillary nucleus) and in other area of the rat brain such as the dentate gyrus, CA2 and CA3 regions of the hippocampal formation, thalamic regions, and several nuclei within the brain stem. Moreover, the finding that GHSR-1a is also observed in the anterior lobe of the pituitary gland is consistent with its role in regulating GH-releasing activity (Guan et al., 1997).

Since circulating ghrelin deriving from the stomach may bind to brain target neurons, it has been demonstrated that it is able to cross the blood-brain barrier in the brain-to-blood direction (Banks, Tschop, Robinson, & Heiman, 2002). Recent findings also revealed that vagotomy prevents peripheral ghrelin's effect on the hypothalamus (Date et al., 2002), suggesting that ghrelin direct effect on the brain (Nakazato et al., 2001; Tschop et al., 2000) may be of intrinsic origin.

The localization of ghrelin in specific brain area, in particular in hypothalamic nuclei, suggests an interaction between this hormone and the pain modulation system. In fact, it was found that ghrelin directly induces neuropeptide Y (NPY) neurons to release NPY (Cowley et al., 2003;

Korbonits, Goldstone, Gueorguiev, & Grossman, 2004), a neuropeptide highly expressed throughout the central and peripheral nervous systems that mediates several physiologic activities including the nociceptive process at the level of the spinal cord (Gibbs, Flores, & Hargreaves, 2004). It has also been observed that ghrelin hypothalamic neurons innervate other peptidergic systems such as proopiomelanocortin (POMC) neurons (Cowley et al., 2003). β-endorphin resulting from POMC gene plays an important role in the descending antinociceptive pathway (Sibilia et al., 2006; Y. G. Sun, Lundeberg, & Yu, 2003). Moreover, ghrelin increases the levels of hypothalamic nitric oxide (NO) synthase (Gaskin, Farr, Banks, Kumar, & Morley, 2003). It is well know that neuronal NO modulates the antinociceptive effect of endogenous opioids by activating μ-opioid receptors; it is therefore possible that ghrelin enhances the antinociceptive effects of endogenous opioids via NO pathway. In addition, it has been observed that ghrelin decreases serotonin release, a modulator of pain and analgesia, (Andersen & Dafny, 1983) in hypothalamus (Brunetti et al., 2002) and raphe nucleus (Carlini et al., 2004).

Several studies showed that ghrelin has also an anti-inflammatory activity by decreasing the expression level of the proinflammatory cytokines IL-1β, IL-6 and TNF-α (Dixit et al., 2004) by lymphocytes and monocytes. It is known that these cytokines contribute to central and peripheral inflammatory pain hypersensitivity (Samad et al., 2001). The antihyperalgesic and neuroprotective effect of ghrelin may therefore be due to the prevention of the production of these proinflammatory cytokines (Dixit et al., 2004; Guneli, Kazikdas, & Kolatan, 2007; Moon, Kim, Hwang, & Park, 2009; Theil et al., 2009; Wang, Bansal, Falk, Ljubanovic, & Schrier, 2009). Finally, ghrelin may exert antinociceptive effects also by directly increasing inhibitory (GABAergic/glycinergic) neurotransmission in a subset of deep dorsal horn neurons, mainly localized in the medial aspect of laminae IV-VI (Vergnano et al., 2008). All these observations suggest a role of ghrelin as a peptide participating in the inhibitory control of pain in pathological states.

Ghrelin has also been shown to promote neurogenesis in several area of the brain. Indeed, it has been demonstrated that ghrelin increases neural cell proliferation in cultured neuronal precursor cells from the fetal spinal cord (Sato et al., 2006), but these effect is greater when the cells are taken from E17 embryos instead of P2 pups (Inoue, Nakahara, Kangawa, & Murakami, 2010). This effect is promoted by both acylated and unacylated ghrelin, suggesting that ghrelin acts through both a GHSR-dependent and GHSR-independent mechanisms to mediate neurogenesis of the embryonic spinal cord.

Moreover, systemic administration of ghrelin increases neurogenesis in the dorsal motor nucleus of the vagus (DMNV) and in the nucleus of the solitary tract (NTS) following vagotomy: ghrelin produced a significant increase in BrdU incorporation both *in vivo* (increases vagotomy-

induced BrdU incorporation in the DMNV and NTS in adult rats) and *in vitro* (cultured DMNV and NTS neurons respond to ghrelin with increase BrdU incorporation), suggesting its potential to promote neuronal development and regeneration (W. Zhang, Hu, Lin, Fan, & Mulholland, 2005; W. Zhang et al., 2004).

Finally, recent studies demonstrated that ghrelin is also involved in hippocampal neurogenesis. Indeed, mice treated with ghrelin showed increased BrdU incorporation and doublecortin-positive neuroblasts in the subgranular zone (SGZ) of the dentate gyrus, and this number was significantly reduced after anti-ghrelin antibody treatment (Moon, Kim, Hwang, & Park, 2009). Moreover, ghrelin knockout mice resulted in reduced numbers of BrdU-positive cells, immature neurons and newly generated neurons in the SGZ, while ghrelin treatment restored these cell numbers to those of wild-type (E. Li et al., 2013). Finally, ghrelin stimulates increased incorporation of ^{3}H-thymidine in adult rat hippocampal progenitor cells, indicating an increased cell proliferation (Johansson et al., 2008).

Intracerebroventricular (icv) administration of ghrelin in the central nervous system induces anxiogenic effects (elevated plus maze test) and increases memory retention in rats (step down test) (Carlini et al., 2002; Carlini et al., 2004), suggesting that ghrelin influences several biochemical processes in the hippocampus. Additionally, in a neonatal rat model with experimental unilateral hypoxic-ischemic injury, icv injections of the synthetic peptidic GH-secretagogue hexarelin significantly reduced the area of injury in the cerebral cortex, hippocampus and thalamus demonstrating a neuroprotective effect of the hormone *in vivo* (Brywe et al., 2005).

More recent studies indicate that ghrelin exerts neuroprotective effects also against chronic glutamate toxicity. Indeed, in an *in vitro* study with a model of excitotoxic motoneuron degeneration of organotypic spinal cord cultures, it has been observed that treatment with ghrelin significantly decreased motoneuron loss by preventing microglial activation in the spinal cord (Lee et al., 2012). Moreover, ghrelin protects spinal cord motoneurons after glutamate excitotoxicity also through the activation of extracellular signal-regulated kinase (ERK) 1/2 and phosphatidylinositol-3-kinase (PI3K)/Akt/glycogen synthase kinase (GSK)-3β pathways (Lim, Lee, Li, Kim, & Park, 2011). Finally, ghrelin has a neuroprotective effect in neurodegenerative diseases such as Parkinson's disease, where it functions as a microglia-deactivating factor (Moon, Kim, Hwang, Seo et al., 2009).

Zhang and colleagues (Q. Zhang et al., 2012) demonstrated the acute effect of ghrelin on ischemia/reperfusion (I/R) injury in the rat spinal cord. Their results suggest that ghrelin administration may inhibit spinal I/R injury thanks to the anti-apoptotic properties of the ghrelin

mechanism that inhibits the apoptosis molecules in the mitochondrial pathway and activates endogenous protective molecules.

Little is known about ghrelin activities in peripheral nervous system. Erriquez et al (2009) showed that DRG cells express GHSR-1a and that ghrelin induces a change in cytosolic calcium concentration in both glia and neurons of embryonic chick DRG (Erriquez et al., 2009).

Daily administration of ghrelin to rats subjected to chronic constriction injury (CCI) of the sciatic nerve improved the histological appearance of the nerve. Moreover, ghrelin prevented mechanical hyperalgesia in CCI rats in a dose-related manner (Guneli et al., 2010).

4. Overexpression of ghrelin promotes motor nerve function recovery after traumatic injury

So far, no information is available about the effects of GHR on peripheral nerve regeneration. Therefore, in order to evaluate if ghrelin can act on neuromuscular recovery, a pilot study was performed in our laboratory on 4 adult male FVB1 WT and 4 FVB1 *Myh6/Ghrl* transgenic mice.

Transgenic FVB1 animals were obtained by cloning the murine ghrelin gene (*Ghrl*) under control of the cardiac promoter sequences of the βMHC 3' UTR and the first three exons of the αMyHC isoform (De Acetis et al., 2005; Porporato et al., 2013). Phenotypical characterization and experiments were carried out on hemizygote animals.

Under deep anesthesia, the median nerve of the left forelimb was approached from the axillary region to the elbow with a longitudinal skin approach and, under operative microscope, was carefully exposed and cut. Transected median nerve was immediately repaired by means of a termino-terminal suture. In order to prevent interferences with the grasping test, the contralateral median nerve was transected at the middle third of the brachium and its proximal stump was sutured in the Pectoralis Major muscle to avoid spontaneous reinnervation.

Functional recovery was assessed by grasping test every 5 days for 70 days after surgery. For the control value, the grasping test was performed the day before the operation. Preliminary results on functional recovery showed that neuromuscular recovery was significantly faster in *Myh6/Ghrl* mice compared to WT mice. Moreover, after 40 days from the injury, WT value was still statistically different compared to the pre-injury value, whereas *Myh6/Ghrl* value was similar to the pre-injury value, showing that the overexpression of *Ghrl*, and the resulting higher levels of circulating unacylated ghrelin, sped up the motor recovery after injury. Finally, at the end of the

experiment (day-70) the two experimental groups reached value similar to the pre-injury values (figure 1).

(INSERT FIGURE 1 HERE)

These first data suggest that this molecule can have an effect also in promoting axonal regeneration after nerve injury and repair. Further studies are needed to understand where and how ghrelin acts.

5. Conclusions

Today, no pharmacological approach has still being introduced in the clinics with the goal of improving posttraumatic neuromuscular recovery. Among the various molecules that are currently being explored in experimental models with such a goal, the circulating peptide hormone ghrelin is receiving increasing interest.

In fact, literature review provided in this paper shows that ghrelin has both a regeneration promoting effect in the central nervous system and a preventive effect against skeletal muscle atrophy. Preliminary data from our laboratory also suggested that this hormone can have an effect in promoting regeneration after nerve injury and repair.

The putative positive effects of ghrelin at all the various structural levels involved in posttraumatic neuromuscular recovery, namely the central nervous system, the peripheral nerve, and the target skeletal muscle, make this molecule an ideal candidate as a therapeutic agent for improving this complex and multilevel regenerative process.

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FIGURE LEGEND

Figure 1

Functional recovery assessment by grasping test after termino-terminal median nerve repair in Wild Type (WT) and *Myh6/Ghrl* transgenic (Tg) mice. Functional recovery is faster in Tg mice (* $p \leq 0.05$ WT *vs*. Tg). After 40 days WT value was still statistically different compared to the pre-injury value, whereas Tg value was similar to the pre-injury value ($ $p \leq 0.05$ WT *vs*. pre-injury).