

Obestatin signaling controls Schwann cells and axonal transport to counteract neuromuscular synaptic loss in skeletal muscle

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Abstract

Background.

Injuries to the peripheral nerve system are common conditions, with broad spectrum of symptoms depending on the impaired nerves and severity of damage. Although peripheral nervous system retains a remarkable ability for regeneration, it is estimated that less than ten percent of patients fully recover function after nerve injury and the available treatments remain suboptimal. Here, we identify a role for the obestatin/GPR39 system in the regulation of the Schwann cell plasticity as well as in the preservation of neuromuscular synapses in the course of nerve repair.

Methods.

Utilizing a compression model of sciatic nerve injury, axonotmesis, we assessed the obestatin-related regenerative response in the peripheral nerve system. The role of the obestatin/GPR39 system was further evaluated on immortalized rat Schwann cells, IFRS1, and the model of neuronal differentiation, PC12 cells. The interactions between SCs and neurons was evaluated using a co-culture system that combine the SC cell line IFRS1 and the NGF-primed PC12.

Results.

Obestatin signaling directs proliferation and migration of Schwann cells that sustain axonal regrowth and later remyelinate regenerated axons. We provide evidence supporting the preservation of skeletal muscle by the maintenance of neuromuscular synapses through the axonal regulation of calpain-calpastatin proteolytic system. This encompasses the control of skeletal muscle homeostasis by regulation of the ubiquitin proteasome system and the autophagy machinery.

Conclusions.

These results provide important insights into how the obestatin/GPR39 system promotes nerve repair through integration of multiple molecular cues of neuron-Schwann cells crosstalk aimed to promote axon growth and guide axons back to their targets.

Background

Peripheral nerve injury represents a health problem that often leads to significant functional impairment and permanent disability. Despite advanced nerve repair techniques, full functional recovery is not achieved in most patients [1]. Therefore, there is an unmet need for therapeutic or adjunctive strategies that promote the functional recovery in nerve injury patients [2]. Intriguingly, the adult peripheral nervous system (PNS) retains significant regenerative potential [3]. Following lesion to the nerve, the axons distal

to the injury site degenerates; notwithstanding, damaged peripheral axons are able to regrow and reinnervate their targets [3]. This process is closely related to the exceptional plasticity of the PNS glia, the Schwann cells (SCs) [4, 5]. In intact nerves, SCs are present in two differentiated states, either myelinating large-caliber axons (myelinating SCs) or ensheathing groups of small-caliber axons in Remak bundles (nonmyelinating SCs). Following injury, SCs respond to nerve injury with a unique regenerative program, characterized by the ability to dedifferentiate to an unmyelinating progenitor state that facilitates axonal regeneration and repair. SCs acquire an array of new phenotypes including the secretion of neurotrophic factors to promote axonal survival, the clearance of myelin debris, the expression of axonal guidance and adhesive cues to generate a favorable environment for axonal regrowth, the initiation of an inflammatory response to promote wound healing, and the proliferation to replace lost cells [4]. Dedifferentiation is a multifaced process regulated by the interplay of cell-intrinsic programs and cell-extrinsic signals. After nerve damage, neuronal degeneration relieves prodifferentiative axonal signals, thereby triggering SC dedifferentiation through activation of cell-intrinsic transcriptional programs. Extrinsic signals from the microenvironment superimpose on these programs in a context-dependent manner to adapt SC function to the specific repair requirements of their surrounding tissue. In fact, SCs maintain their adhesive epithelial characteristics in the proximal region. By contrast, in the distal region, SCs express genes associated with stem-cell characteristics and acquisition of mesenchymal traits, being even more pronounced in the wound region induced by localized TGF β signaling [6]. These changes provide a conducive substrate for successful nerve regeneration by which dedifferentiated SCs extend through the nerve bridge as multicellular cords that guide the regrowing axons [4, 7, 8]. Curiously, despite the SC-associated features, the complete functional recovery after peripheral nerve trauma is rare. Furthermore, the myelin sheath thickness of regenerated axons remains usually reduced [3, 9–18].

Despite the research on nerve repair and therapeutic development is widely studied, the translation of these ideas into clinical applications has not taken place at the same rate [19]. Nowadays the treatment of choice for peripheral nerve injury is advanced microsurgical end-to-end repair with tensionless epineural sutures or nerve autografts when end-to-end anastomosis is not possible [20, 21]. However, microsurgical approaches fail to address the complex cellular and molecular events associated with peripheral nerve injury. Several treatment strategies have been employed to enhance the recovery process, including pharmacological, electrical, and cell-based therapies [22–25]. Despite each of these approaches is considered promising method, little clinical benefit has been reported. The development of possible treatment strategies should be addressed on the bases on how the particular communication between the target organ and the neural cell body is orchestrated and how might be deregulated to lead to the breakdown of myelin sheath and axons (Wallerian degeneration) [1, 2]. Indeed, the redifferentiation of SCs and the efficient remyelination of new axons happen in a critical time window, in which SCs are susceptible to growth-factor stimulation [15, 16, 18].

In previous works, we demonstrated the therapeutic potential of the obestatin/GPR39 system, an autocrine/paracrine system, to regulate skeletal muscle repair [26–30]. Obestatin, a 23-amino acid peptide derived from a polypeptide called preproghrelin, exerts an autocrine anabolic function in skeletal muscle to control the myogenic programme through the G protein-coupled receptor GPR39 [26].

Mechanistically, obestatin enhances muscle regeneration by regulating multiple steps of myogenesis: myoblast proliferation, cell cycle exit, differentiation, and recruitment-to fuse and form multinucleated hypertrophic myotubes. This action is coordinated by the interplay between G protein-dependent and β -arrestin-dependent mechanisms [28]. In addition, obestatin participates in the specification of muscle fiber identity by inducing skeletal muscle remodeling toward an oxidative phenotype [31]. Significantly, the obestatin/GPR39 system counteracts deregulations in proteostasis, e.g. those associated to glucocorticoid-induced myotube atrophy, and to restore efficient basal homeostasis [32]. In cell transplantation therapy, obestatin not only enhances the efficiency of engraftment but also facilitates an even distribution of myoblasts within the host muscle by enhancing migration [31]. Furthermore, obestatin ameliorate the Duchenne muscular dystrophy phenotype [30]. Interestingly enough, obestatin triggers an up-regulation of the neuromuscular junction (NMJ) genes. These data position obestatin as a potential Duchenne therapeutic candidate not only as an ameliorative strategy to slow the muscle damage but also as part of combinatorial treatment strategies.

In this study, we identify critical roles for the obestatin/GPR39 system in regulating the plasticity of SCs, as well as in preserving neuromuscular synapses during PNS regeneration. We find that obestatin directs different stepwise from the repair program of SCs, including proliferation and migration, that guide regrowing axons and later remyelinate regenerated axons. Importantly, we provide evidence supporting the preservation of skeletal muscle by the maintenance of neuromuscular synapses probably through the axonal regulation of mitofusin 2 (Mfn2) and calpain-calpastatin proteolytic system. This provides the necessary signals and spatial cues for the regulation of autophagy and ubiquitin-proteasome systems in skeletal muscle. These results provide the first attractive framework for further understanding the role of the obestatin/GPR39 system in preserving neuromuscular synapses and serve as a therapeutic approach to skeletal muscle atrophy.

Material And Methods

Materials

Rat/mouse obestatin was obtained from BCN Peptides (Barcelona, ES). Antibodies used are listed in Table S1. All other chemical reagents were from Sigma Chemical Co. (St. Louis, MO, US).

Animals

All work involving animals was carried out according to the University of Santiago de Compostela guidelines for animal handling and care. Sprague-Daley rats were purchased from the Central Animal Facility of the University of Santiago de Compostela (Santiago de Compostela, ES).

Cell lines

PC-12 cells were obtained from ATCC (Rockville, MD, US). IFRS1 cell line was kindly provided by Dr Kazunori Sango (Tokyo Metropolitan Institute of Medical Medicine, Tokyo, JP).

Cell culture

PC-12 cells were maintained in RPMI 1640 (Lonza, Basel, CH), and supplemented with 10% (v/v) fetal bovine serum (FBS; GE Healthcare Life Sciences, UT, US), 5% (v/v) heat inactivated horse serum (HS; Gibco, NY, US) and 1% glutamine/penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, US). PC12 cells were seeded on type I collagen-coated plates in DMEM (Lonza, Basel, CH) supplemented with 5% FBS (v/v), 100 U/mL penicillin and 100 mg/mL streptomycin for 24–48 h. Neuronal differentiation was initiated by switching to differentiation medium [DM; DMEM supplemented with N2 supplement (Millipore-Merck, Darmstadt, DE), 50 µg/mL ascorbic acid (Sigma-Aldrich, MO, US), 50 ng/mL recombinant rat β -nerve growth factor (NGF) (R&D Systems Inc., Minneapolis, MN, US)] for 7 days (d). IFRS1 cell line was maintained in DMEM supplemented with 5% (v/v) FBS, 20 ng/mL recombinant human heregulin- β III (Merck, Darmstadt, DE), 5 µM forskolin (Abcam, Cambridge, UK), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, MO, US). IFRS1 and PC12 coculture model was performed as described previously with slight modifications [33]. Briefly, after differentiation of PC12 cells (3×10^2 cells/cm²) for 7d following the procedure described above, IFRS1 cells suspended in DMEM/5% FBS (v/v) (1.5×10^4 cells/cm²) were added to NGF-primed PC12 cell. After incubation in DMEM/5% FBS (v/v) for 48 hours (h), co-cultured PC12-IFRS1 cells were fed every 48 h in myelination medium [MM; DMEM containing B27 supplement (Gibco, NY, US), 50 µg/mL ascorbic acid, 10 ng/mL NGF, and 25 ng/mL neuregulin-1 type III] and maintained for up to 21d. Obestatin stimulation (200 nM) was performed in cocultured PC12-IFRS1 cells every 48 h in MM.

Cell proliferation assays

IFRS1 cells were seeded at 1.5×10^4 cells/cm² in 96-well plates in DMEM/ 5% FBS (v/v). Cells were synchronized by serum-starvation for 16 h and then stimulated with different obestatin doses (25–200 nM) for 24 h. IFRS1 cell proliferation was assessed by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation using the Cell Proliferation ELISA-BrdU kit (Roche Diagnostics, Basel, CH) according to the manufacturer's instructions. Cell proliferation in PC12-IFRS1 coculture was determined by crystal violet staining assay. Treated coculture cells were rinsed with Milli-Q water and then stained with a working solution of 0.5% crystal violet at room temperature for 20 min as previously described [34]. Cells were then washed extensively with Milli-Q water to remove unbound dye and two images of random fields were taken per well using a ZEISS Axio Vert.A1 inverted microscope (Carl Zeiss; Oberkochen, DE). For quantification, methanol was added to the stained culture plates and then analyzed by spectrophotometry at 570 nm using a microplate reader (BioTek; Vermont, US).

Migration assays

IFRS1 cells (2.2×10^4 cells/cm²) were seeded into the culture-insert 2-well (Ibidi, Martinsried, DE), grown to 100% confluence, and then serum starved for 16 h. Culture insert was removed and cells were stimulated with obestatin (200 nM, 32 h). The progress of migration was photographed immediately after removing the culture insert and at 8, 24, and 32 h post-stimulation using a ZEISS Axio Vert.A1 inverted microscope (Carl Zeiss; Oberkochen, DE). The migration area was measured by tracing along the gap

border using the Fiji analysis software and the wound closure was calculated as follows: Migration (%) = [(Cell gap (t = 0 h)-Cell gap (t = x h))/Cell gap (t = 0 h)]x100. Cell migration was further evaluated by cell-inverted invasion assay as previously described [35]. Rat obestatin (200 nM) was added as the chemoattractant and cells were allowed to migrate into growth factor-reduced Matrigel® (Gibco; NY, US) for 24 h. Cells were stained with calcein-acetoxymethyl ester (Invitrogen, Thermo Fisher Scientific; Waltham, MA, US) and visualized by confocal microscope (Leica TCS SP2, Leica Microsystems AG; Wetzlar, DE). Optical sections were scanned at 5-µm intervals moving up from the underside of the membrane into the Matrigel®. The fluorescence from each optical section was quantified with Fiji Analysis software. 3D-reconstruction was performed using the ImageJ analysis software (Volume Viewer software package).

Immunohistochemistry

Rat dorsal root ganglia (DRG), anterior horn of spinal cord and sciatic nerves were dissected out and fixed in paraformaldehyde (PFA). Samples were then washed in phosphate buffered saline (PBS) and dehydrated. Subsequently, samples were embedded in paraffin and sectioned on a microtome at a thickness of 4 µm. Samples were washed with washing buffer [containing: 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% (v/v) Tween 20] and blocked with peroxidase-blocking reagent (Dako, Agilent, CA, US). Sections were incubated with primary antibodies (Table S1) [diluted in Dako antibody diluent Dako, Agilent, CA, US] and EnVision™ FLEX/HRP (Dako, Agilent, CA, US) was used as the detection system. 3,3'-Diaminobenzidine [DAB-tetrahydrochloride (Dako, Agilent, CA, US) was used as chromogen, and nuclei were counterstain with Harris haematoxylin solution (PanReac, Castellar del Valles, ES). Finally, sections were mounted with Entellan mounting medium (Merck, Darmstadt, DE) for imaging. For analysis of muscle, tibialis anterior (TA) were mounted in tissue freezing medium [gum tragacanth (Santa Cruz Biotech Inc., Dallas, TX, US)] and snap frozen in nitrogen-cooled isopentane. The sections, 10 µm thick, were mounted on Histobond Adhesion Microslides (Marienfeld, Lauda-Königshofen, DE). For the haematoxylin/eosin (HE) staining, serial cryostat sections were stained following a standard protocol. Compiled images were used to reconstruct a view of the entire TA muscle. This compilation was used for quantification of cross-sectional area using Fiji and Image J64 analysis software.

Immunofluorescence

For analysis of cultured cells, the PC12 and IFRS1 cells were co-cultured on coverslips. Cells were fixed in PFA, washed, permeabilized, and blocked with normal goat serum (Invitrogen, Thermo Fisher Scientific; MA, US). Samples were stained with primary antibodies and then incubated with specie-specific secondary antibodies. 4',6-Diamidino-2-phenylindole (DAPI) was used to counterstain the cell nuclei (Life Technologies, Thermo Fisher Scientific; MA, US). Analysis of NMJs were performed following the protocol previously described [36]. Gastrocnemius muscles were longitudinally sectioned into 20 µm cryosections. Muscle sections were permeabilized and blocked with PBT [1% (v/v) Triton X-100, 1% (v/v) Tween-20, 5% (v/v) heat inactivated goat serum, 0.2% (w/v) BSA in PBS], and incubated with primary antibodies diluted in PBT overnight at 4°C, washed with PBS and then incubated with an appropriate secondary antibody and a-bungarotoxin. For each rat, at least 150 NMJs were counted for quantification. The digital images

were acquired with a Zeiss AxioVert A1 microscope. Confocal images were captured with a with a Leica TCS-SP8 spectral confocal microscope (Leica Microsystems, Heidelberg, DE). Colocalization analysis was conducted using NIH ImageJ software (Coloc 2 plugin). Overlap coefficient according to Pearson and Manders ($m1$ and $m2$) were used to determine the colocalization [37].

Immunoblot analysis

Protein was isolated using ice-cold radioimmunoprecipitation assay buffer [RIPA, containing: 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) Na-deoxycholate, protease inhibitor cocktail (Sigma Chemical, MO, US), phosphatase inhibitor cocktail (Sigma Chemical, MO, US)]. The lysates were clarified by centrifugation ($18000 \times g$, 15 min, 4 °C), and the protein concentration was quantified using the QuantiPro™ BCA assay kit (Sigma Chemical, MO, US). For immunoblotting, equal amounts of protein were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, IL, US).

Staining of lipids with Oil Red O

Lipids was quantitatively evaluated using Oil Red O staining. Briefly, PC12-IFRS1 coculture cells were fixed with buffered paraformaldehyde-PBS, rinsed three times with Milli-Q water and then with 60% isopropanol. Lipid droplets were then stained with Oil Red O. For quantification, Oil Red O was extracted using isopropanol and absorbance was measured at 520 nm by spectrophotometry.

Peripheral nerve surgery

Animals, male Sprague-Daley rats (250 g), were assigned to one of the following experimental groups ($n = 8$ per group): 1) sham group, rats without sciatic nerve injury ($n = 8$); 2) control group, rats with sciatic nerve injury under vehicle administration [0.9% NaCl (w/v), saline; $n = 8$]; and, 3) obestatin-treated group, rats with sciatic nerve injury under obestatin administration (500 nmol/kg body weight, $n = 8$) into the target sciatic nerve every 48 h during 12d. The rats were anaesthetized with ketamine/xylazine cocktail followed by shaving the leg area. With the support of a heating pad, the anesthetized rat was placed in dorsal recumbency and the paws were gently immobilized on the surgery platform with a piece of tape with the paw-palm facing up. An incision was made on the skin, along the proximal half of the line between the trochanter major and the knee joint. The sciatic nerve was exposed through gluteal muscle splitting incision, through which the overlying latialis and biceps femoris muscles were separated using a pair of straight forceps. The sciatic nerve was crushed 1 cm proximal to the division of the sciatic nerve into the tibial and common peroneal nerves. The crush was made using a Dumont N7 negative action curved tweezers (Dumont, Montignez, CH), taking care to do it within the same section, located 0.5 cm from the end of the tweezer and exerting a constant pressure for 10 seconds. The damaged area was then encapsulated with the catheter port. The inlet end of the catheter port was inserted subcutaneously to the neck and extracted through and small incision and secured with silk suture. Obestatin or saline (0.9% w/vehicle) was administrated through the inlet end catheter. At the indicated time points post-surgery for each experiment described, animals were euthanized by cervical dislocation.

Footprint Test

Footprint analyses were performed using a walking track (11.5×7.5 cm) with an enclosed chamber. The hind paws of rats were dipped into black non-toxic paint. Rats were placed on the runway covered by white paper where they ran toward the enclosed dark box. The parameters measured normal (N) and experimental (E) feet were: a) longitudinal distance between the tip of longest toe and the heel (PL); b) the cross-sectional distance between the first and the fifth toes (TS); and c) the cross-sectional distance between the second and the fourth toes (ITS). The sciatic functional index (SFI) was calculated using the formula previously described [38, 39].

Statistical analysis

All values are presented as mean $\pm$ SEM. ROUT test ($Q = 5\%$) was performed to detect the presence of outliers. Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilief P value was performed to evaluate normality. Un-paired Student t-test was used to compare means of two groups with normal distribution. Mann-Whitney test was used to compare medians of two groups with non-normal distribution. Anova with Dunnett multiple comparison was used to compare means of two or more groups with normal distribution. Kruskal-Wallis with Dunn's multiple comparison was used to compare means of two or more groups with non-normal distribution. *, **, ***, and **** denotes $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

Results

The expression of the obestatin/GPR39 system in the peripheral nerve system. In adult rat sciatic nerve, GPR39 localized primarily to SCs of myelinated fibers, while obestatin showed a diffuse expression pattern (Fig. 1a). Strong GPR39 immunoreactivity was associated with the plasma membrane and ectoplasm of larger-sized DRG neurons, whereas more diffuse cytoplasmic immunostaining, with occasional granular staining of the plasma membrane, appeared in the smaller-sized DRG neurons (Fig. 1b). Diffuse cytoplasmic immunostaining was observed for obestatin in DRG neurons (Fig. 1b). In rat anterior horn of spinal cord, GPR39 immunostaining was observed in the motor neurons and glia, whereas obestatin immunoreactivity was only observed in motor neurons (Fig. 1c). The expression pattern of obestatin/GPR39 in PNS and, in particular in SCs, prompted us to examine its expression pattern in response to nerve injury. We initially performed a spatial analysis of the obestatin/GPR39 system on a recovered rat sciatic nerve after a complete transection (Fig. 1d). We found that, by 12d after the cut, proximal and distal compartments showed GPR39 positivity, which was strongly associated to longitudinal cell columns, the bands of Büngner, in the distal part (Fig. 1d). Of note, obestatin expression varied significantly between distal and proximal areas revealing an increase in the distal compared to proximal stump (Fig. 1d). Using cell-type-specific markers to identify obestatin-positive cells in the distal nerve stump by immunofluorescence, we identified SCs, Sox10 positive cells (Sox10⁺), expressing high levels of obestatin (Fig. 2). In contrast, macrophages (F4/80), endothelial cells (CD31) and regenerating axons (neurofilament, NF) in the distal nerve stump did not express levels of obestatin (Fig. 2), ruling out

their implications as sources for the expression of this peptide in the distal nerve stump. Thus, nerve injury was associated with increased obestatin levels in the distal SCs consistent with a role as chemoattractant to guide cells and their accompanying axons out of the nerve stumps and across the bridge during peripheral nerve regeneration. We also confirmed obestatin expression on cultured IFRS1 cells (data not shown).

Obestatin signaling drives peripheral nerve regeneration. To assess the obestatin-related regenerative response in the peripheral nerve system we employed a standard compression model of sciatic nerve injury (axonotmesis), an injury model that allows to study the interaction among regenerative axon with both the SCs and basal laminae [40]. Obestatin (500 nM/Kg body weight per 48 h for 12d) or vehicle [0.9% NaCl (w/v), corresponding volume] was administered into crush-injured sciatic nerve by using a catheter port (Fig. 3a). Motor function was assessed with standard SFI analysis (see details in the Footprint Test from the Method section), using measurements of total footprint length (PL), toe spread (TS), and intermediate toe spread (ITS). Walking footprint patterns and quantification of SFI revealed functional recovery of obestatin-treated rats as compared to control rats at 3, 6 and 12d post-injury (Fig. 3b, right and left panels, respectively). At 21d post-injury, there was a significant increase in the expression of the neuronal outgrowth marker, growth-associated protein-43 (GAP43), in the crush and distal sites of obestatin-treated rats compared to control (Fig. 3c). Within 12d of injury, longitudinal sections of regenerating nerves exhibited a significant increase in the NF-stained positive axons (NF⁺) in the nerve segment distal to the crush site of the obestatin-treated nerves compared to control (Fig. 3d). There was also an increase for the SC marker, myelin basic protein (MBP) in the distal nerve segment (Fig. 3e). In this segment, the number of MBP⁺ longitudinal tubular structures were augmented noticeably in obestatin-treated rats at 12d post-injury, showing a decrease of ellipsoid bodies also termed as degeneration chambers (Fig. 3e). Consistently, immunoblot analysis demonstrated the upregulation of GAP43 and makers of myelinating SCs such as MBP, myelin-associated glycoprotein (MAG), myelin protein zero (MPZ), POU protein Oct6 (OCT6) and early growth response 2 (EGR2) in obestatin-treated rats relative to control animals (Fig. 3f). Together, these data imply that obestatin signaling favors the ability of dedifferentiated SCs to switch back to a myelinating phenotype on contact with regenerated axons.

Because myelination and axonal integrity play a critical role in the functional recovery after PNS injury, the obestatin effects in these parameters was evaluated taking the advantage by using the immortalized rat SC, IFRS1, and the model of neuronal differentiation, PC12, cells. As shown in Figure S1a, obestatin led to a significant dose-dependent upregulation of IFRS1 proliferation rates when compared to control. Added to mitogenic effect, a wound-healing assay comparing the migration of IFRS1 cells at different time points revealed that obestatin acted as a mobility signal for SCs (Figure S1b). Furthermore, an inverted Boyden chamber migration assay supported the role of obestatin (200 nM) as chemotactic signal for SCs (Figure S1c). IFRS1 cells were able to migrate through the membrane, mimicking basement membrane invasion and invade into the Matrigel[®] as an extracellular matrix when obestatin (200 nM) was applied on top of the Matrigel[®] as a chemoattractant (Figure S1c). With respect to PC12 cells, the role

of obestatin signaling was analyzed in differentiated PC12 cells (dPC12 cells: 7d DM-primed PC12 cells). Obestatin-treated dPC12 cells showed a reduction in the number of neurites in accordance with the decrease in the percentage of differentiated cells (Figure S2a and S2b, respectively). Of note, obestatin increased mean neurite length (Figure S2c) and polarity index (Figure S2e), with no significant effect on principal neurite length (Figure S2d) by promoting the conversion from the multipolar morphology to the bipolar one. These observations were consistent with the early signaling pathways activated by obestatin/GPR39 system in PC12 cells. It is known that epidermal growth factor (EGF) and NGF show opposing actions to proliferate or differentiate which are dictated by the duration of extracellular signal regulated kinase 1/2 (ERK1/2) signaling in PC12 cells (41). When PC12 cells were stimulated with obestatin (200 nM) led to transient phosphorylation of the EGF receptor (EGFR) at Y1068 and sustained phosphorylation response of NGF receptor (TRKA) at Y490 (Figure S2f). The interplay among G protein-coupled GPR39, EGFR and TRKA activated by obestatin signaling network triggered sustained ERK1/2 phosphorylation at T202/Y204 [pERK1/2(T202/Y204)] (Figure S2g). Similarly, obestatin (200 nM) evoked sustained Akt phosphorylation at S473 [pAkt(S473)] (Figure S2g). This temporal specification rewires the system toward cell differentiation in response to obestatin signaling. This observation further supports the importance of activation duration to yield the correct cell fate decision [41–47].

The role of the obestatin/GPR39 system in the interactions between SCs and neurons was established using a co-culture system that combine the SC cell line IFRS1 and the NGF-primed PC12 (33). In this PC12-IFRS1 model, PC12 cells acquired a neuronal phenotype, demonstrated by morphological activation with neurite elongation in close contact with SCs (Figure S3a). Importantly, MBP staining was mainly detected in areas corresponding to IFRS1 cells closely attached to the neurites emerging from PC12 cells, demonstrating a stable and effective SC-axon interaction (Figure S3b). In this model, cell proliferation assay showed a significant increase in obestatin-treated cells under myelinating conditions (200 nM; Fig. 4a). To discriminate between IFRS1 and PC12 cell proliferation, both cell types were cultured for 7d in myelinating conditions and the proliferation was analyzed separately. As expected, obestatin (200 nM) led to IFRS1 cell proliferation (Fig. 4b), with no evidence in PC12 cells (Fig. 4c). Additionally, we observed a marked increase in the number of IFRS1 cell clusters in the obestatin-treated co-culture compared to control (200 nM; Fig. 4d). Analysis of cluster area revealed an increase in obestatin-treated co-culture (Fig. 4e). Likewise, transect measurements across clusters revealed an increase in cell density in obestatin-treated co-cultures compared to control (Fig. 4f). Finally, analysis of several key myelin proteins was supportive of a global increase in myelination levels upon obestatin treatment. In particular, obestatin increased the expression of MPZ, MBP and MAG in IFRS1 cells within 21d after coculture (Fig. 4g). These changes were directly correlated with the increase in the lipid biosynthesis and the expression of sterol regulatory element-binding protein (SREBP) cleavage activating protein (SCAP) (Fig. 3h and 3i, respectively) at 21d post-co-culture. High levels of MBP were evident in obestatin-treated co-culture with colocalization to MBP⁺-SCs and tyrosine hydrolase (TH)-stained axons (Fig. 4j). This result was further analyzed by Manders *m2* coefficient, where an increase in overlap of PC12 cells by SCs was evident in obestatin-treated group compared with control (Fig. 4j). Accordingly, the transects taken perpendicularly along PC12 neurites demonstrated an increment of the MBP⁺-SC density in the obestatin-

treated group compared to control (Fig. 4k). Taken together, these findings support the role that obestatin signaling exerts in the myelination and axonal integrity.

Obestatin-mediated regeneration of sciatic nerve reverses skeletal muscle atrophy. Since decline in neuromuscular innervation is known as an endogenous cause of muscle atrophy, we first measured the TA, extensor digitorum longus (EDL), gastrocnemius (GM) and soleus muscle weights to assess muscle atrophy. 21d after injury, we observed loss of muscle mass in the control group (sciatic nerve injury control) that was counteracted by obestatin treatment in all tested muscles (Fig. 5a). Using HE stained cross sections of the TA muscles from each group fiber, cross-sectional areas (CSA) were measured and compared among groups (Fig. 5b). Remarkable, CSA analysis showed that the treated-to-control ratio of all fibers was 43% larger in obestatin-treated group ($3665 \pm 49 \mu\text{m}^2$) than in control ($2564 \pm 35 \mu\text{m}^2$) at 21d post-injury (Fig. 5b). The myofiber area distribution showed that high percentage of the individual fibers in the obestatin-treated group had fiber areas between 2500–3250 μm^2 , whereas most of the fibers in the control group had areas between 1500–2250 μm^2 (Fig. 5b). In terms of protein degradation, obestatin treatment led to a significant decrease in the expression of the ubiquitin E3-ligases MAFbx and MuRF1 (Fig. 5c). The two major signaling pathways regulating skeletal muscle atrophy program are the Forkhead box O (FoxO) transcription factors and histone deacetylase proteins (HDAC) in several pathophysiological conditions, including neurogenic atrophy, muscle disuse, and cancer cachexia [48, 49]. In fact, the down-regulation of MAFbx and MuRF1 expression was concomitant with increased phosphorylation of FoxO3a at T32 and FoxO1 at T24 but did not change basal FoxO4 phosphorylation at T28 in the obestatin-treated group (Fig. 5d). However, the upregulation of HDAC4, which represses Dach2, a negative regulator of myogenin, resulted in myogenin expression in both obestatin and control groups (Fig. 5d) ruling out its implication in this proteolytic pathway. It is known the role that the mTORC1 pathway plays in stimulating protein synthesis in skeletal muscle [49]. After 21d of nerve injury, levels of the phosphorylated form of mTOR at S2448 increased in TA muscle in both control and obestatin-treated groups (Fig. 5e). We then focused our attention on the two best-characterized mTORC1 targets, the ribosomal protein S6 [downstream target of the serine/threonine kinase p70S6K1 (S6K1)], and the eIF4E-binding protein 1 (4E-BP1) [50, 51]. Levels of phosphorylated form of S6 at S240/244 were increased in control group in TA muscle, but this effect was significantly lower in obestatin-treated animals (Fig. 5e). Interestingly, an analysis of the phospho-forms of 4E-BP1 showed a strong increase in the hyperphosphorylated form of 4E-BP1, designated as g form, at T37/46 residues in obestatin-treated group, but not in control group (Fig. 5e). The interplay between the ubiquitin-proteasome and autophagy-lysosome systems determines the regulation proteostasis as well as its extent in the context of different catabolic or anabolic conditions [52]. In this particular case, autophagy induction was reduced in TA muscles in obestatin-treated animals, as shown by limited increase in the lipid modified form of LC3, referred as LC3II, and increased levels of p62 and cathepsin-L (mature form), in relation to control group (Fig. 5f). Taken together, these data support a model whereby the interplay between mTOR and FoxO regulates the ubiquitin-proteasome and the autophagy-lysosome systems, and the signaling associated with protein translation in response to the obestatin/GPR39 system in the target muscle of the regenerating nerve.

Obestatin signaling delays axonal degeneration and neuromuscular synaptic loss upon nerve injury. To test if the function of obestatin in preventing muscle wasting was related to the inhibition of axonal degeneration, we analyzed proteins involved in mitogen-activated protein kinase (MAPK)/glycogen synthase kinase 3 β (GSK3 β) signaling, apoptosis, and cytoskeleton formation in the sciatic nerve (Fig. 6a). Compared to control sciatic nerves, pERK1/2(T202/Y204), a pathway classically associated with neurite outgrowth [53, 54], was significantly increased in obestatin-treated sciatic nerves at 12d post-injury (Fig. 6a). The activation of ERK1/2 was concurrent to the inactivation of GSK3 β , estimated as GSK3 β phosphorylation at S21/9 [pGSK3 β (S21/9); Fig. 6a], a pathway required for transforming neurons into a regenerative state upon injury [55]. Remarkably, obestatin-treated sciatic nerves exhibited significant increase of c-Jun-N-terminal kinase (JNK) phosphorylation at T183/Y185 [pJNK(T183/Y185)], a signaling node involved in axonal growth and regeneration (Fig. 6a) [56]. Unexpectedly, the proapoptotic BH3-only protein Bim and the apoptosis regulator Bax were increased in response to obestatin signaling, suggestive of apoptosis (Fig. 6a). However, the cleaved and active caspase 3 expression, major effector in neurite degeneration, was clearly downregulated in obestatin-treated group, especially when compared with control group (Fig. 6a). Despite the activation of these apoptosome pathway components, the survival promoting kinases associated to obestatin signaling are effective enough for regulating caspase 3 expression and thus inhibiting neuronal apoptosis. Finally, obestatin-treated group showed significantly increase of axonal proteins such as NF medium (NF-M) and NF light (NF-L), with significantly upregulation of α -tubulin and β III-tubulin (Fig. 6a). NFs not only provide structural support for neurons, but also interacts with many proteins and organelles, including tubulin, to establish a regionally specialized network that serves as a docking platform to organize other organelles and proteins [57]. These results imply that obestatin may protect neuromuscular synapses through mechanisms involving the inhibition of axonal degeneration.

Calpastatin, an endogenous inhibitor of calcium-dependent cysteine protease calpain, is involved in protein degradation, neuromuscular function regulation [38], and axon survival [58]. A noteworthy increase of the calpastatin level was observed in obestatin-treated sciatic nerves 12d post-injury (Fig. 6b). Interestingly, the change of calpastatin level correlated with upregulation of the calpain-2 level in the obestatin-treated group, while calpain-1 remained unchanged (Fig. 6b). In contrast, the calpain-1 level was increased in the control group, whereas calpastatin and calpain-2 remained unchanged (Fig. 6b). In both cases, the calpastatin level and its balance with calpain activity were key determinants of how calpains are regulated. Additionally, obestatin-treated sciatic nerves exhibited significant increase in the mitochondrial outer membrane protein Mfn2 levels, a protein involved in the axonal transport of calpastatin to protect NMJs [38]. Mfn2 is enriched at the junction between the endoplasmic reticulum (ER) and mitochondria, which is known as the mitochondria-associated ER membranes (MAMs) [59]. Consistent with Mfn2 findings, an increase in the ER marker calnexin level was noted in obestatin-treated sciatic nerves (Fig. 6b). Thus, Mfn2 upregulation delayed onset and progression in this model of sciatic nerve injury by raising calpastatin levels, essential for axonal survival. Indeed, the change of calpastatin levels correlated with the cytoskeletal protein levels, specifically NF-M, NF-L, β III-tubulin and α -tubulin. Additionally, the nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) levels showed to be

regulated by obestatin signaling (Fig. 6b) supporting the view that Nmnat1 inhibits an upstream step leading to calpastatin depletion.

The role of Mfn2 and mitochondria in the axonal transport of calpastatin is sufficient to inhibit localized calpain activation, axon degradation, neuromuscular synaptic loss and muscle atrophy upon nerve injury [38]. Remarkably, the ratio of acetylcholine receptor (AChR)-rich postsynaptic sites on myofibers was increased in TA muscles after sciatic nerve injury under obestatin administration (Fig. 7a). Agrin, muscle-specific kinase (MuSK), and Wnt family member 3 (Wnt3) are key regulators of NMJs. Agrin and Wnt3 are secreted by motor neurons, whereas MuSK is mainly expressed in skeletal muscles [60]. In skeletal muscles, obestatin-treated rats exhibited significant increase in the expression levels of Agrin and Musk proteins but not Wnt3 protein at 12d post-injury (Fig. 7b). In sciatic nerve, obestatin-treated rats showed significant increased levels of Agrin and Wnt3 proteins related to control (Figs. 7b). Remarkably, in rats with sciatic nerve injury under obestatin administration, NMJ innervation, estimated by Pearson and Manders correlation coefficients between synaptic vesicle glycoprotein 2A (SV2) and AChR (α-bungarotoxin for motor endplates), was sustained at a level comparable with this of rats with no sciatic nerve injury (Fig. 7c). These data provide evidence supporting the role of obestatin signaling in preserving neuromuscular synapse loss upon nerve injury through the regulation of Mfn2-mediated calpastatin transport and, thus, the calpain-calpastatin proteolytic system.

Discussion

The use of obestatin peptide as a regenerative agent for traumatic peripheral nerve damage offers a novel approach to address an unmet medical need. Beyond its established role in myogenesis, obestatin singularly enhance both the speed and extent of recovery of motor behavior after crush injury to the sciatic nerve, as analyzed by sciatic functional test. These benefits were associated with an efficient regulation of the SC plasticity to direct the dedifferentiation, axonal regrowth, and remyelination. Indeed, we provide evidence supporting the preservation of skeletal muscle mass by the maintenance of neuromuscular synapses probably through the axonal regulation of Mfn2 and calpain-calpastatin proteolytic system. This includes the control of muscle homeostasis by regulation of the autophagy and ubiquitin-proteasome systems in a coordinated manner involving distinct set of effector proteins that ultimately affect FoxO transcription factors. This study provides the first attractive framework for further understanding the role of the obestatin/GPR39 system in the axonal transport and myelination and possibly other systems to inhibit neuromuscular synaptic loss upon nerve injury.

Mature differentiated SCs retain a high degree of plasticity throughout adult life and upon injury shed their myelin sheaths and dedifferentiate to a progenitor/stem cell-like state [4, 61]. Dedifferentiation is a multifaceted process controlled by the interplay of cell-intrinsic programs and cell-extrinsic signals. After nerve damage, neuronal degeneration releases prodifferentiative axonal signals that trigger SC dedifferentiation through activation of cell-intrinsic transcriptional programs. Extrinsic signals from the microenvironment then superimpose on these programs in a context-dependent manner to adapt SC function to the precise repair necessities [62]. From the data presented thus far, obestatin might be

incorporated into the list of regulatory factors that control SC phenotype. The fact that obestatin expression increases in the distal region where dedifferentiated SCs switch off the myelination program, supports a regulatory role not only on SC proliferation to replace lost cells but also on the collective migration of SCs to drive nerve repair. Further support is demonstrated by the effect on the de-novo myelination of axonal networks, with the resulting exit from the cell cycle and expression of specific transcription factors to the myelinating cell type. The increased expression of pro-myelinating factors, such as Oct6 and EGR2 [63, 64], reinforces this suggestion. This transcriptional feedforward network ultimately leads to the extensive production of lipids and myelin proteins in vitro and vivo, such as MBP, MAG, MPZ and SCAP [61, 63, 65], that ensure an appropriate ensheathment and myelination of axons [66–68]. Thus, obestatin may be included to the group of extrinsic and/or intrinsic signals that modulate and balance negative and positive factors to control SC proliferation and their transition to a differentiating state during peripheral myelination [4, 12]. Both processes should operate properly to generate sufficient SCs for subsequent differentiation and myelination of peripheral nerve regeneration and repair [12, 63]. Defects in SC generation and differentiation during regeneration may cause a failure in myelinogenesis, contributing to SC deterioration associated with motor disabilities. However, this is unlikely, as we observe functional recovery after obestatin treatment in peripheral nerve injury. In any event, it is quite remarkable how a single molecule is capable to have such a role in regulating SCs at so many different points of their lineage.

Following peripheral nerve injury, obestatin expression is up-regulated in the distal nerve stumps. This type of signal may be understood as a trophic factor to promote axonal survival and regulate neuronal elements. In PC12 cells, obestatin was shown to enhance prolonged ERK1/2 and Akt activities by crosstalk with the receptor tyrosine kinase EGFR and TRKA to direct polarization and orientation of these cells under obestatin signaling. Although it is somewhat difficult to extrapolate such actions on PC12 cells to in vivo system, this is consistent with the idea that cells enter into a non-stable, transitional state from which they can be polarized for axonal outgrowth and switch back to a multipolar state. This fact would be related to a stereotypical spatial organization and dynamics of the axon cytoskeleton that ensure the elongation and steering of injured peripheral axon [69]. Accordingly, obestatin shows to activate significant pro-growth modules, such as JNK and PI3K-GSK3 pathways [55, 70], as well as to upregulate growth-associated proteins, including GAP43 [56, 71], and cytoskeletal components, such as α -tubulin, β -tubulin and NFs [72]. Regenerating axons need to carry out de novo growth to reach their targets. This regrowth period demands a constant supply of cytoskeleton, organelles and other building blocks to the growing tip. Up-regulation of Mfn2 and calpastatin expression might be indicative of a control on localized calpain activation, axon degradation, and neuromuscular synaptic loss. MAMs and their resident protein Mfn2 allowed mitochondrial co-transport of calpastatin to inhibit localized calpain activation and axon degradation. The co-transport depends on Mfn2 ability to form endoplasmic reticulum-mitochondria tethers to deliver MAMs and MAM-residing proteins like calpastatin to distal nerve endings. The delivery of calpastatin to distal nerve endings leads to the localized inhibition of calpain that finally prevents axon degeneration [38, 59, 73]. Indeed, up-regulation of calnexin was observed in obestatin-treated rats, denoting co-enrichment of MAMs. Therefore, here we uncover an unexpected

function of obestatin signaling on mitochondria-MAMs-mediated trafficking for calpastatin axonal transport. Added to the Mfn2-mediated calpastatin transport to regulate the calpain-calpastatin proteolytic system, Nmnat1 was observed to be up-regulated by obestatin. Increase of Nmnat1 levels causes downstream inhibition of sterile- α and Toll/interleukin 1 receptor motif containing protein 1 (SARM1), probably as a result of decreased levels of nicotinamide mononucleotide but potentially also in other ways [74]. The combined effect of increased Nmnat1 and decreased activation of SARM1 leads to a great increase in axonal nicotinamide adenine dinucleotide, which may itself prevent axon degeneration through ATP synthesis [75]. Alternatively, other SARM1 substrates or its calcium mobilizing products could be important for the later stages of Wallerian axon degeneration [76–79]. While this and other aspects are under investigation, this study proposes a mechanism by which obestatin preserves axonal integrity by the activation of mitochondrial-mediated axonal transport of calpastatin and the inhibition of pro-degenerative molecule SARM1. Obestatin-regulated axon regeneration is therefore likely conferred by coordinated actions of multiple components and pathways functioning together in the complex injured environment.

Neuronal Mfn2 upregulation appears to be essential to maintain neuromuscular endplates and to prevent muscle atrophy after nerve injury [38]. Indeed, this study shows that obestatin signaling protects neuromuscular synaptic loss and, therefore, counteracts atrophy of hindlimb skeletal muscles innervated by motor neurons. In particular in TA muscle, this action involves the decreased activity of the ubiquitin/proteasome system, with downregulation of the E3 ubiquitin-ligases MAFbx and MuRF1 under the control of FoxO transcription factors, FoxO1 and FoxO3. Additionally, obestatin limits muscle atrophy by promoting protein synthesis and inhibiting autophagy. Protein synthesis is related to the eIF4E availability, through phosphorylation of 4E-BP1, which shifts the balance from protein degradation to protein synthesis in obestatin-treated rats. Importantly, we unveil a determinant function of obestatin signaling in neuromuscular integrity that allow to regulate muscle homeostasis via specific activation of the Akt-mTORC1 axis. Although mTORC1 becomes activated in control muscle, constant activation of mTORC1 triggers feedback inhibition of Akt [80]. This fact determines a key difference between groups that establishes the activity of the ubiquitin/proteasome system by regulation of E3 ubiquitin-ligases under the control of HDAC4/myogenin and FoxO transcription factors [80–84]. Interestingly, the activation of S6 alone, or lack of mTOR signaling to 4E-BP1 only, is not sufficient to increase muscle mass in control muscle. These results underlie the importance of the 4E-BP1/eIF4E in stimulating protein synthesis and support the idea that both mTOR downstream targets mediate independently AKT-induced muscle growth [85]. Overall, our study provides strong evidence supporting the critical role of the obestatin/GPR39 system in the axonal transport of calpastatin, MAMs, and probably other cytosolic proteins to inhibit localized calpain activation, axon degradation, neuromuscular synaptic loss and, consequently, skeletal muscle atrophy.

During reinnervation, axons continue to grow beyond the boundary of denervated NMJ where they can reach another synaptic site, leading, along with the axonal reinnervation, to the polyinnervation of NMJs [86]. In our in vivo model, the obestatin/GPR39 system exerts a role in this polyinnervation affecting the ratio between the AchR structures and myofibers and thus improvement of the expression of components

and clustering of NMJ upon nerve injury. At molecular level, this is in accordance with the results obtained from the expression of Agrin, at pre- and post-synaptic terminal, and the receptor tyrosine kinase MuSK, at post-synaptic endplate. Agrin acts by binding to its receptor low-density lipoprotein receptor-related protein 4 (Lrp4), a member of the low-density lipoprotein receptor family, in muscle to activate MuSK [87, 88]. Both Lrp4 and MuSK are critically important for Agrin-induced AChR clustering, maintenance and further growth [60, 86, 89]. Interestingly, our observations point to Wnt3 as component of obestatin signaling at pre-synaptical terminal. Wnt3 promotes the number and size of Agrin-dependent AChR clusters [90–93]. This mechanism seems to ensure proper apposition between the presynaptic and postsynaptic apparatus, as well as innervation of each muscle. Indeed, control rats displayed greatly decreased NMJ innervation, yet obestatin-treated group regained NMJ innervation reaching levels comparable with sham controls. Combined, these all enable the structural and functional integrity of the NMJ to be maintained.

Conclusions

Although the obestatin/GPR39 system has been thought of as a regulator of myogenesis, this study highlights its involvement in crucial aspects of peripheral nerve regeneration. Obestatin signaling orchestrates SC plasticity to promote and guide axonal repair. On the other hand, obestatin preserve neuromuscular synapses by regulating the axonal transport of Mfn2, calpastatin, and possibly other cytosolic proteins to inhibit localized calpain activation, axon degradation, and neuromuscular synaptic loss. In summary, glial obestatin-mediated paracrine and autocrine stimulation constitutes an attractive target for therapeutic approaches of wide range of diseases including, but not limited to, aging- and disease-related skeletal muscle atrophy.

Abbreviations

AChR: acetylcholine receptor; CSA: cross-sectional areas; d: days; DRG: rat dorsal root ganglia; EGR2: early growth response 2; EGFG: epidermal growth factor; EGFR: EGF receptor; EDL: extensor digitorum longus; ER: endoplasmic reticulum; FBS: fetal bovine serum; FoxO: Forkhead box O; GAP43: growth-associated protein-43; GM: gastrocnemius; GSK3 β : glycogen synthase kinase 3 β ; HDAC: histone deacetylase proteins; HE: haematoxylin/eosin; h: hours ITS: intermediate toe spread; JNK: c-Jun-N-terminal kinase; Lrp4: lipoprotein receptor-related protein 4; MAG: myelin-associated glycoprotein; MAPK: mitogen-activated protein kinase; MAMs: mitochondria-associated ER membranes; MBP: myelin basic protein; Mfn2: mitofusin 2; MM: myelination medium; MPZ: myelin protein zero; MuSK: muscle-specific kinase; NF: neurofilament; NF-M: NF medium; NF-L: NF light; Nmnat1: nicotinamide mononucleotide adenylyl-transferase 1; NGF: β -nerve growth factor; NMJ: neuromuscular junction; OCT6: POU protein Oct6; PBS: phosphate buffered saline; PFA: paraformaldehyde; PL: footprint length; PNS: peripheral nervous system; SCs: Schwann cells; SFI: sciatic function index; SCAP: sterol regulatory element-binding cleavage activating protein; SREBP: sterol regulatory element-binding protein; SV2: synaptic vesicle glycoprotein

2A; TA: tibialis anterior; TH: tyrosine hydrolase; TRKA: NGF receptor; TS: toe spread; Wnt3: Wnt family member 3.

Declarations

Ethical approval

Experiments were granted by Santiago de Compostela University Animal Welfare and Ethical Review Board according to the guidelines of the Spanish Royal Decree 53/2013, Directive 2010/63/EU and FELASA Guidelines. Bioethical experimental procedure committee code: 15010/17/005.

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

Not applicable.

Competing of interests

The authors declare no competing interests.

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Author's Contributions

Y.P. and J.P.C. conceived the project. R.G., J.L.R., J.P.C. and Y.P. designed the experiments. J.L.R., A.S.T. and J.G.S. performed the in vivo experiments. A.S.T. and S.L.L. performed the in vitro experiments. A.S.T. and C.S.M. performed immunohistochemistry and immunofluorescence assays. A.S.T., R.G., J.L.R., J.P.C. and Y.P. analyzed the data. Y.P. and J.P.C. wrote the manuscript with critical review and input from all other co-authors. All authors read and approved the final manuscript

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Figures

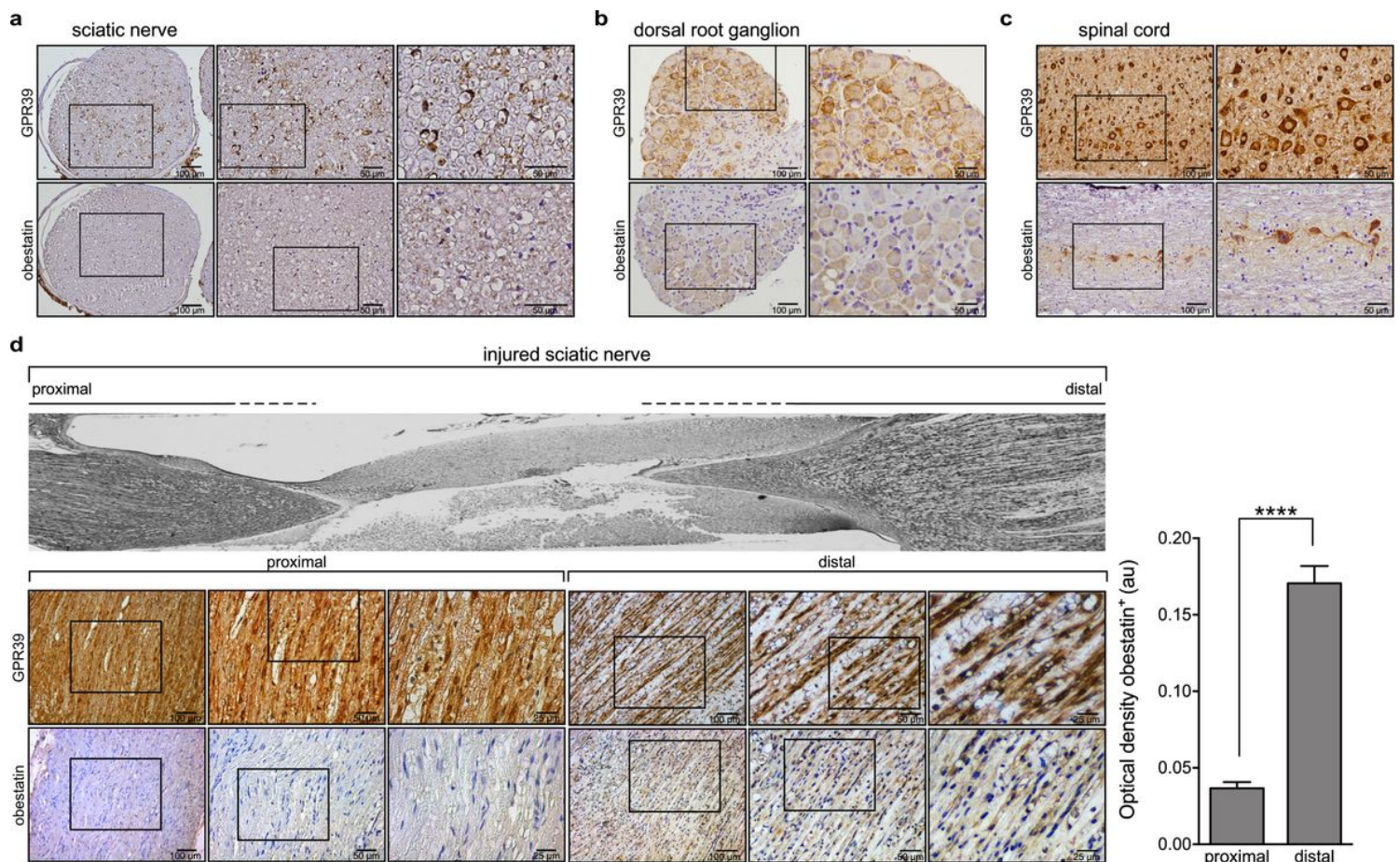


Figure 1

Molecular signatures of the obestatin/GPR39 system in the PNS. Representative images of immunohistochemical detection of GPR39 (upper panel) and obestatin (lower panel) in a cross-section of rat sciatic nerve (a), DRGs (b) and anterior horn of spinal cord (c). (d) Left panel, example immunohistochemical detection of GPR39 and obestatin of a regenerated rat sciatic nerve collected 12d post-transection. Images are representative of the proximal and distal stump areas. Right panel, quantification of obestatin expression (optical density) in the proximal and distal nerve stumps at 12d post-transection (mean $\pm$ SEM; n=6, **** P < 0.0001).

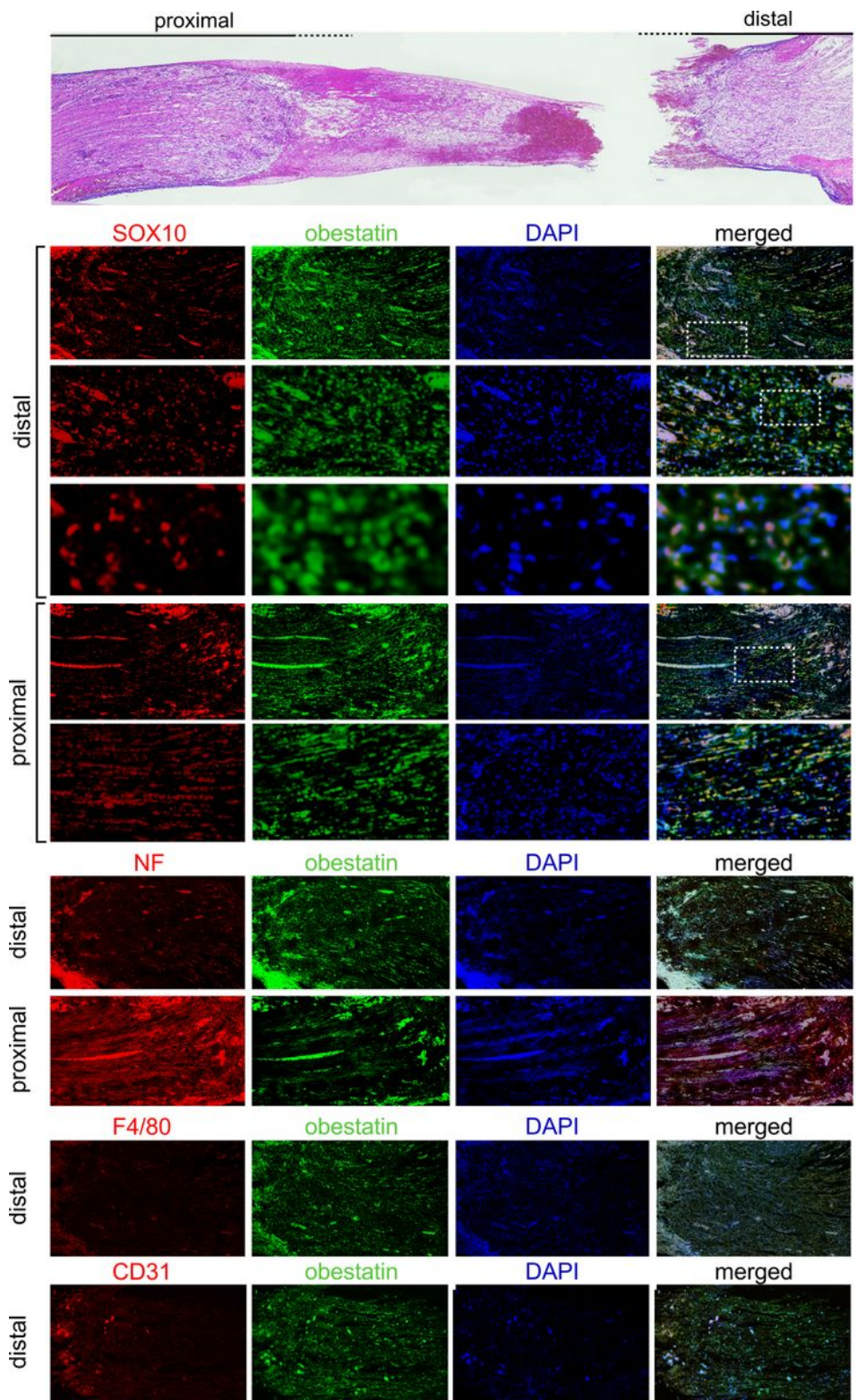


Figure 2

Up-regulation of obestatin levels in the distal stumps is associated to SCs. Representative images of longitudinal sections of injured sciatic nerves immunostained for SCs (SOX10+, red), NF (NF+, red), macrophages (F4/80+, red), or endothelial cells (CD31+, red), and obestatin (obestatin+, green) at 7d after transection. Nuclei were counterstained with DAPI (blue). Images are representative of the proximal and distal stump areas.

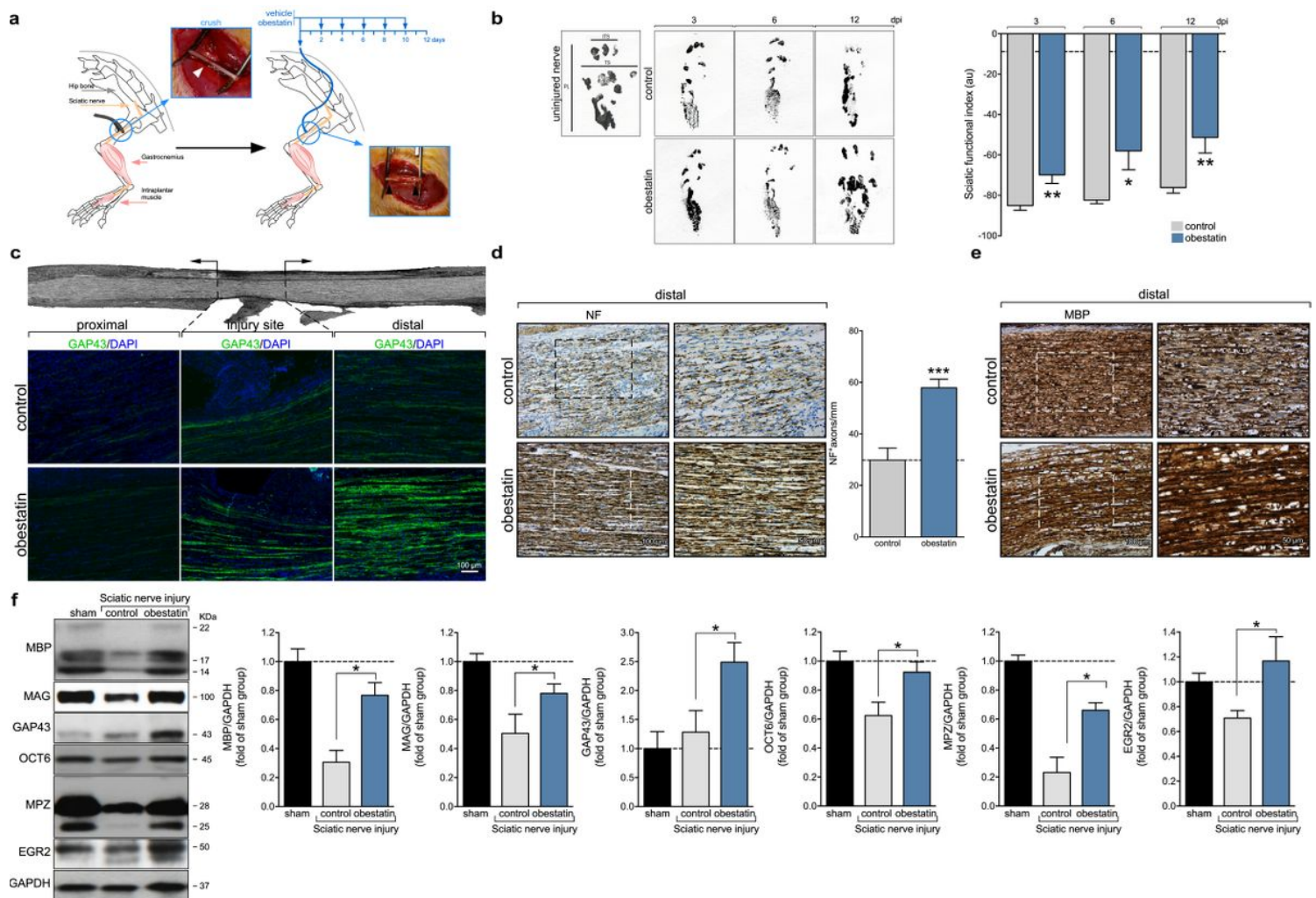


Figure 3

Obestatin programs SCs and axon integrity to drive peripheral nerve regeneration. (a) Schematic diagrams showing the rat model of peripheral nerve injury. Rats underwent sciatic nerve crush, which leaves epineurium and perineurium intact to support nerve growth. Obestatin (500 nM/Kg body weight) was administered into crush-injured sciatic nerve by catheter port. Rats were treated every 48h with a single injection of obestatin (500 nM/Kg body weight) for 12d. (b) Left panel, representative walking footprint patterns of non-injured and injured rats after administration of obestatin (200 nmol/kg body weight/48h during 3-, 6- or 12d; n= 8 per time point) or vehicle (saline; control). The values used to calculate the SFI were indicated (PL, ITS, and TS). Right panel, SFI showing the recovery of injured sciatic nerve at indicated time point. Data were represented as mean $\pm$ SEM (*, **P < 0.05 and P < 0.01 respectively). (c) Immunofluorescence of longitudinal sections of the sciatic nerves at 12d after crush injury using GAP-43 antibody. Upper panel, representative image of a crush-injured sciatic nerve. (d) Left panel, immunohistochemistry using NF antibody of longitudinal sections of the distal nerve segment from control- or obestatin-treated rats at 12d post-nerve injury. Right panel, quantification of the number of NF+ axons per mm from control- or obestatin-treated sciatic nerves at 12d post-nerve injury. Data were represented as mean $\pm$ SEM (***P < 0.001). (e) Immunohistochemistry of longitudinal sections of the distal nerve segment from control- or obestatin-treated rats using MBP antibody at 12d post-nerve injury.

(f) Immunoblot analysis of MBP, MAG, GAP43 OCT6, MPZ and EGR2 in crush-injured sciatic nerves from control- or obestatin-treated sciatic nerves at 12d post-nerve injury. Contralateral sciatic nerves were used as sham group. Immunoblots are representative of the mean value (n=8). Data were expressed as mean $\pm$ SEM obtained from intensity scans (*P < 0.05).

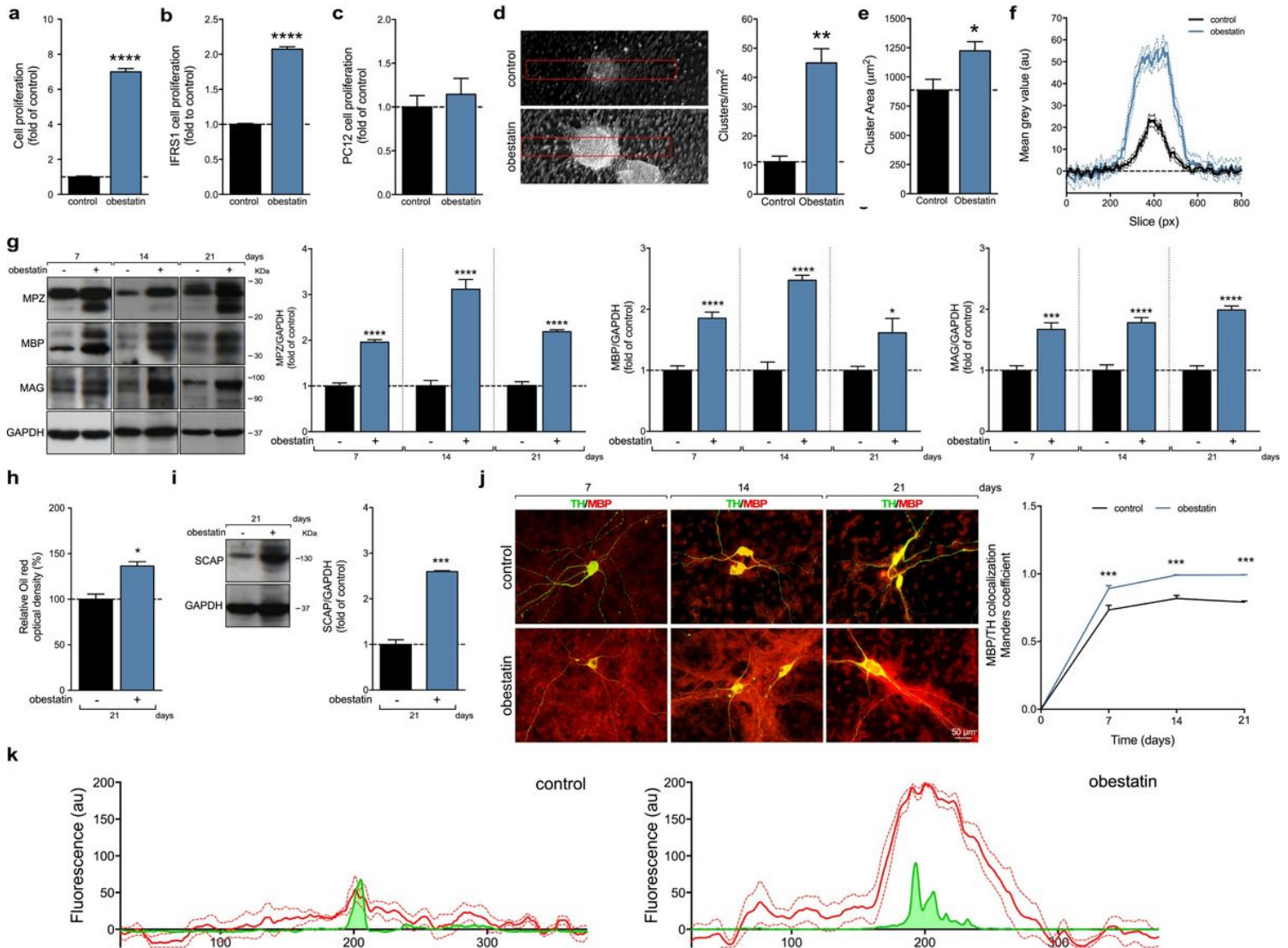


Figure 4

Obestatin signaling drives SC proliferation, and myelination in a co-culture system that combine IFRS1 and NGF-primed PC12 cell lines. (a) Quantification of cell proliferation by violet crystal incorporation in PC12-IFRS1 coculture under stimulation with obestatin (200 nM each 72h, 19d; n=6) or vehicle (saline each 72h, 19d; n=6). Data were expressed as mean $\pm$ SEM (****P < 0.0001). (b) Quantification of cell proliferation by violet crystal incorporation in IFRS1 cells in myelinating conditions under stimulation with obestatin (200 nM, 7d; n=6) or vehicle (saline, 7d; n=6). (c) Direct PC12 cell counting after 7d of obestatin (200 nM, 7d; n=6) or vehicle (saline, 7d; n=6) under myelinating conditions. (d) Left panel, representative phase-contrast images of NGF-differentiated PC12 and IFRS-1 SC cells under stimulation with obestatin (200 nM) or vehicle (saline) at 21d. The red boxes represent transects analyzed for the cell density calculation. Right panel, quantification of the number of the co-cultured PC12-IFRS1 cell clusters per area

(mm²) under stimulation with obestatin (200 nM) or vehicle (saline) at 21d. (e) Quantification of the surface area of the co-cultured PC12-IFRS1 cell clusters under stimulation with obestatin (200 nM) or vehicle (saline) at 21d. (f) Cell density in transects taken through the co-cultured PC12-IFRS1 cell clusters under stimulation with obestatin (200 nM) or vehicle (saline) at 21d. From d-f, data were expressed as mean $\pm$ SEM (n=23 per group; *P < 0.05 and **P < 0.01). (g) Immunoblot analysis of MPZ, MBP and MAG in co-cultured PC12-IFRS1 cells after treatment with obestatin (200 nM) or vehicle (saline) at 7-, 14- and 21-d post-co-culture. Protein level was expressed as fold of control. Data were expressed as mean $\pm$ SEM obtained from intensity scans (n=6; *P < 0.05; ****P < 0.0001). (h) Quantification of lipid accumulation by Oil red staining in co-cultured PC12-IFRS1 cells after treatment with obestatin (200 nM) or vehicle (saline) at 21d. Results are expressed as fold of lipid accumulation over control (n=6; mean $\pm$ SEM; *P < 0.05). (i) Immunoblot analysis of SCAP in co-cultured PC12-IFRS1 cells after treatment with obestatin (200 nM) or vehicle (saline) at 21d post-co-culture. Protein level was expressed as fold of control. Data were expressed as mean $\pm$ SEM obtained from intensity scans (n=6; *** P < 0.001). (j) Right panel, immunofluorescence analysis of myelination in co-cultured PC12-IFRS1 cells after treatment with obestatin (200 nM) or vehicle (saline) at 7-, 14-, and 21-d post-co-culture. Left panel, quantitative MBP/TH colocalization analysis using Manders M2 coefficient from immunofluorescence microscopy images obtained from co-cultured PC12-IFRS1 cells after treatment with obestatin (200 nM) or vehicle (saline) at 7-, 14-, and 21-d (n=6; mean $\pm$ SEM; *** P < 0.001). (k) Quantification of IFRS1 cell density (red line) around PC12 neurites (green line) in PC12-IFRS1 co-cultured cells after treatment with obestatin (200 nM) or vehicle (saline) at 21d (n=10; mean $\pm$ SEM). The transects taken perpendicularly along PC12 neurites were analyzed by measuring the fluorescence intensity for TH and MBP in each transect section.

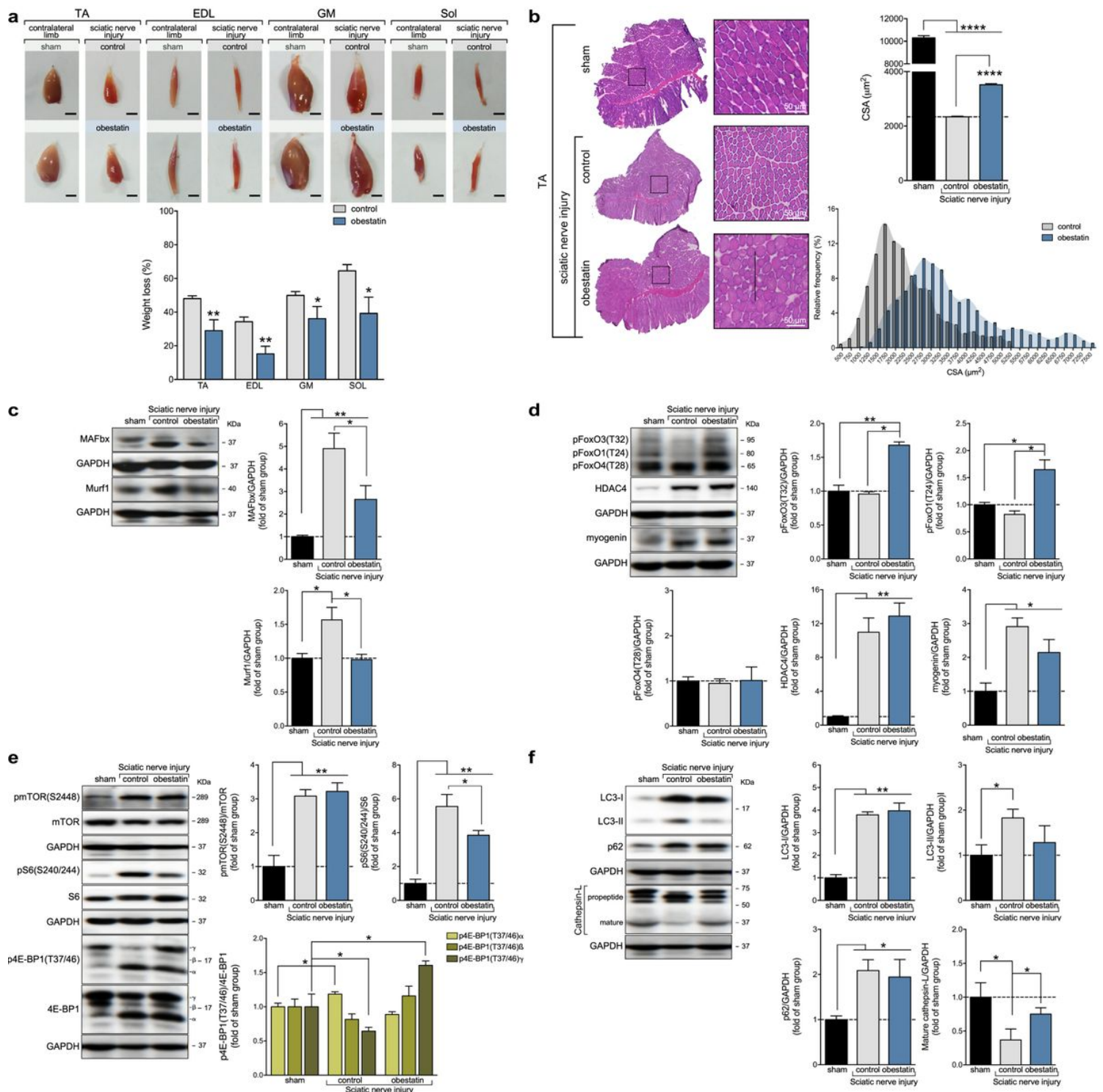


Figure 5

Activation of obestatin signaling in sciatic nerve during regeneration dramatically abolishes skeletal muscle atrophy. (a) Images and quantification of hindlimb skeletal muscles obtained from rats under control- or obestatin-treated sciatic nerves at 12d post-nerve injury ($n=4$ per group; mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$). (b) Left panel, representative HE staining from TA muscles obtained from sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. Right upper panel, cross-sectional area of TA muscle fibers (mean $\pm$ SEM; $n=4$ per group; **** $P < 0.0001$). Right down panel, distribution of fiber

diameter from control- and obestatin-treated rats. Data are expressed as % of myofibres. (c) Immunoblot analysis of MAFbx and MuRF1 in TA muscles from sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. (d) Immunoblot analysis of the phosphorylation partner of FoxOs, HDAC4, and myogenin in TA from sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. (e) Analysis by immunoblot of the phosphorylation partner of mTOR, S6, and 4E-BP1 in TA from sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. (f) Immunoblot analysis of LC3, p62, and Cathepsin-L in TA from sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. From c-f, data were expressed as mean $\pm$ SEM (n=4 animals per group; *P<0.05, **P<0.01) and immunoblots are representative of the mean value.

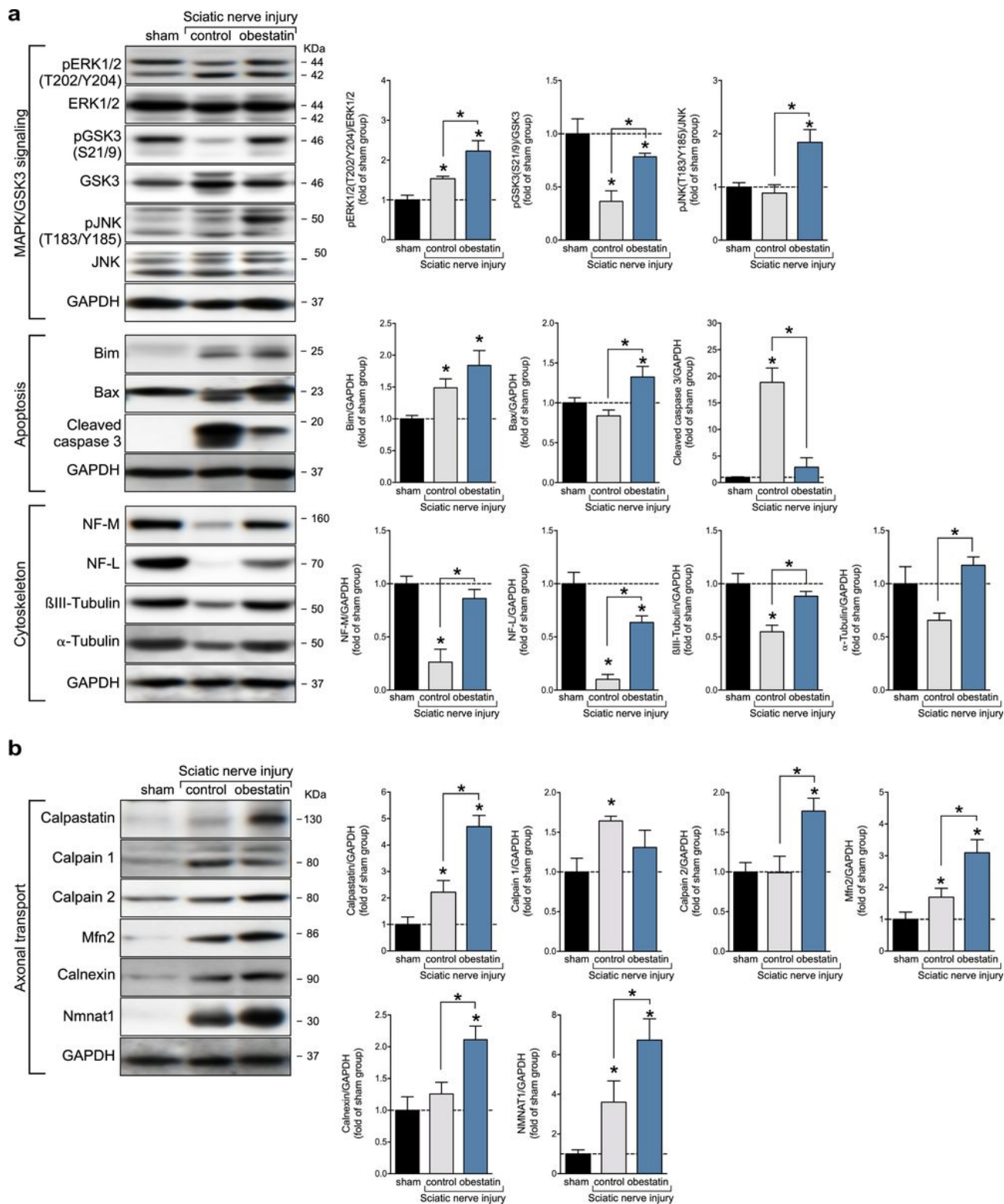


Figure 6

Obestatin signaling protects axon degradation. (a) Analysis by immunoblots of proteins involved in MAPK/GSK3 β signaling (ERK1/2, GSK3, JNK), apoptosis (Bim, Bax, cleaved caspase 3), and cytoskeleton formation (NF-M, NF-L, β III- and α -tubulin) in sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. (b) Immunoblot analysis of proteins involved in axonal transport (Calpastatin, Calpain 1, Calpain 2, Mfn2, Calnexin, and Nmnat1) in sham, vehicle- and obestatin-treated

crush-injured sciatic nerves after 12d post-injury. In a-b, data were expressed as mean $\pm$ SEM (n=4 animals per group; *P<0.05) and immunoblots are representative of the mean value.

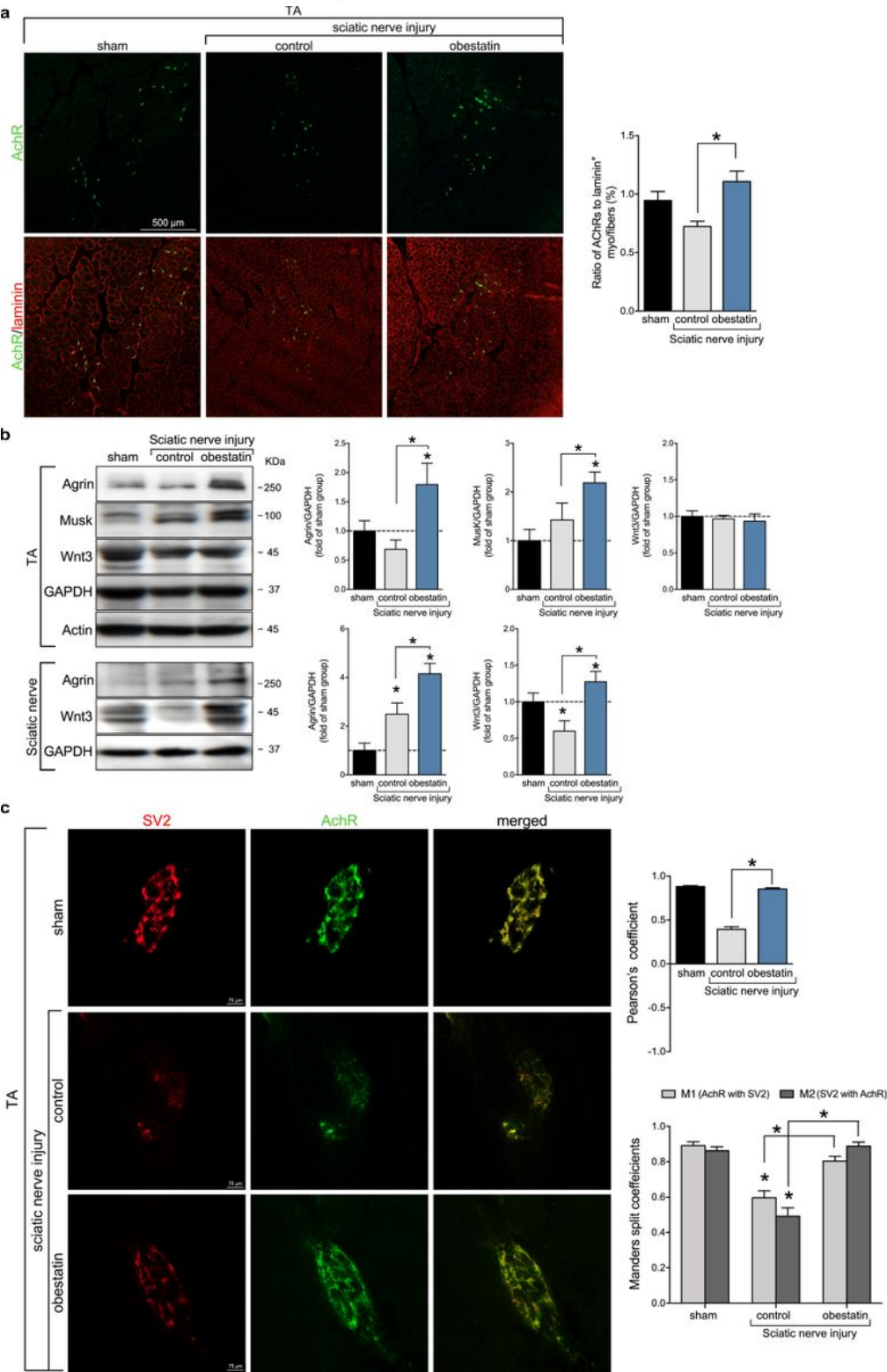


Figure 7

Obestatin signaling in neurons avoids neuromuscular synapse loss. (a) Quantification of AchRs (α -bungarotoxin, green) in TA muscle sections of sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. Data were expressed as ratio of AchRs to laminin positive myofibers (mean $\pm$

SEM; n=4; *P<0.05). (b) Analysis by immunoblots of proteins involved in NMJ in TA muscle (Agrin, Musk, and Wnt3) and sciatic nerve (Agrin, and Wnt3) in sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. Data were expressed as mean $\pm$ SEM (n=4 animals per group; *P<0.05) and immunoblots are representative of the mean value. (c) Representative images (endpoint; left panel) and quantification (Pearson and Manders Split coefficients; right panels) of NMJ innervation by labelling of AchR by α -bungarotoxin (green) and immunofluorescence detection of neurofilaments (SV2) on TA muscle sections of sham, vehicle- and obestatin-treated crush-injured sciatic nerves after 12d post-injury. Data were expressed as mean $\pm$ SEM (n=4 animals per group; *P<0.05).

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