Enzymology:

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Neurolysin Knockout Mice Generation and Initial Phenotype Characterization*

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*Running Title: *Neurolysin KO mice*

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Background: Neurolysin is known to cleave several bioactive peptides *in vitro*.
Results: Neurolysin knockout mice showed increased glucose tolerance, insulin sensitivity and gluconeogenesis, which likely relates to increased expression of both, specific liver mRNAs and intracellular peptides.
Conclusion: Neurolysin plays a role in energy metabolism.
Significance: Neurolysin could be used as a therapeutic target to counteract insulin resistance.

ABSTRACT

The oligopeptidase neurolysin (EC 3.4.24.16; Nln) was first identified in rat brain synaptic membranes and shown to ubiquitously participate in the catabolism of bioactive peptides such as neurotensin and bradykinin. Recently, it was suggested that Nln reduction could improve insulin sensitivity. Here, we have shown that Nln knockout mice (KO) have increased glucose tolerance, insulin sensitivity and gluconeogenesis. KO mice have increased liver mRNA for several genes related to gluconeogenesis. Isotopic label semi-quantitative peptidomic analysis suggests increase in specific intracellular peptides in gastrocnemius and epididymal adipose tissue, which likely is involved with the increased glucose tolerance and insulin

sensitivity in the KO mice. These results suggest the exciting new possibility that Nln is a key enzyme for energy metabolism and could be a novel therapeutic target to improve glucose uptake and insulin sensitivity.

INTRODUCTION

Neurolysin (EC 3.4.24.16; Nln) is a zinc metalloendopeptidase with a conserved HEXXH motif. Nln was first identified in synaptic membranes of the rat brain by the specific cleavage and inactivation of the peptide neurotensin at the Pro-Tyr bond (1-3). Nln is a monomeric protein with a molecular mass of 78 kDa and 704 amino acids residues, which hydrolyzes only peptides containing from 5-17 amino acids by cleaving at a limited set of sites that are nonetheless diverse in sequence (4-9). The specificity of Nln for small bioactive peptide substrates without bulky secondary and tertiary structures is due to the presence of large structural elements erected over its active site region that allow substrate access only through a deep narrow channel (10). It is well established by biochemical analyses *in vitro* that Nln cleaves a number of bioactive peptides including neurotensin, bradykinin, hemopressin, and several opioid peptides at sequences that vary widely(5,10,11).

Nln is widely distributed in mammalian tissues and is found in different subcellular locations that vary with cell type (12-14). Much of the enzyme is cytosolic, but it also can be secreted or associated with the plasma membrane (12,14), and some of the enzyme is synthesized with a mitochondrial targeting sequence by initiation at an alternative transcription start site (5,15). Light and electron microscopic immunohistochemical studies have indicated that within the brain, Nln is present in both neurons and glia (12-14,16). Studies in the neuroendocrine cell line AtT-20 have shown that Nln is enriched in the regulated secretory pathway (17). Nln was also shown to be released from glial cells in culture (14). The mechanism underlying Nln release is unclear, however, as the enzyme has no signal peptide or a hydrophobic membrane spanning domain (14,18). *In vivo* Nln has been suggested to be implicated in pain control (19-21), blood pressure regulation (22,23), sepsis (24), reproduction (25,26), cancer biology (27), and in the pathogenesis of stroke (28).

Previous studies suggested the involvement of proteases, peptidases, and peptides in metabolic disorders such as insulin resistance, obesity and metabolic syndrome (29-32). For instance, carboxypeptidase E knockout mice ($Cpe^{fat/fat}$) show an obesity phenotype (33,34) and dipeptidyl peptidase-4 (DPP4) deficient mice are resistant to develop adiposity and obesity induced by high-fat diet, and exhibit improved insulin sensitivity (29,30). Nln activity is significantly lower in the fat tissue of transgenic mice that possessed three copies of the angiotensin-converting enzyme (ACE) gene. When these mice were fed a high-fat diet they showed better preserved insulin sensitivity compared with the group containing a single copy of the ACE gene (35). The reduced Nln activity in animals containing three copies of the ACE gene seem to correlate with a distinctive intracellular peptide profile of these animals; these results suggested that intracellular peptides could improve insulin sensitivity in the adipose tissue (35). Moreover, additional peptidomic studies using semi-quantitative liquid chromatographic mass spectrometric (LC-MS/MS) have shown that the intracellular levels of two out of ten intracellular peptides found in the epididymal adipose tissue of animals, were at least two times higher in rats fed with a hypercaloric diet when compared to a control group. Indeed, if added back into 3T3-L1 adipocytes these intracellular peptides significantly increased glucose uptake, corroborating the suggestion that intracellular peptides may be functional and regulate insulin signal transduction and glucose uptake in the adipose tissue (35,36).

Here, Nln knockout mice (KO) were generated to allow further investigation on the biological function of this peptidase in energy metabolism. Our initial phenotype characterization of KO mice seem to corroborate previous suggestions that Nln plays a physiological role in energetic balance increasing glucose tolerance, insulin sensitivity and liver gluconeogenesis. However, although one cannot exclude compensatory mechanisms for a subset of Nln-associated phenotypes, we did not observe a key role of this oligopeptidase in blood pressure regulation.

EXPERIMENTAL PROCEDURES

Generation of Nln knockout mice

The Nln gene-trap knockout mouse strain was generated by C57BL/6 blastocyst micro-injection of genetically modified Embryonic Stem cells (NPX481, 129ola) obtained from Baygenomics through the International Gene-Trap Consortium (IGTC, http://www.genetrap.org/).

The genetically modified Nln allele of the NPX481 strain has the gene-trap vector pGT1dTMpfs inserted between exon 1 and exon 2 (Figure 1A), as evidenced by sequencing 5'RACE cDNA from the original NPX481 ES cell line (CC178547), used to generate this mice strain (A detailed report about this ES cell line can be found at the following web link: http://www.genetrap.org/cgi-bin/annotation.py?cellline=NPX481).

After transmission of the knockout allele from chimera to F1 generation, Nln mice were obtained from heterozygous breeding and the line was further maintained on the mixed background by breeding +/- with +/- animals. To obtain such mice on a pure genetic background, we bred F1 (129/OlaHsd/C57BL/6 background) heterozygous Nln-deficient animals to the inbred C57BL/6 mouse line (Charles River) for 10 generations before using them for experimental investigations.

Mice were maintained in individual ventilated cages (TECNIPLAST DEUTSCHLAND) under standardized conditions with an artificial 12-h dark–light cycle, with free access to standard chow (SSNIFF) and drinking water *ad libitum*. The experiments were performed with adult mice (24-weeks-old). This study was approved by the Ethics Committee of Institute of Biomedical Sciences at the University of São Paulo, protocol register number 44, page 86 in the book 02.

Genotyping

Genotyping of animals was performed using PCR with primer NlnF3 (5'-CGCCTCCTGCACCTACCA), pNlnwtR3 (5'-ATTTGCCAGGTTAAGAGATCG), and pNlnkoR2 (5'-CGTGTCCTACAACACACACTCC). The absence of Nln-transcripts in the liver, muscle, and adipose tissue of Nln-deficient animals was confirmed by qPCR with the primer pair, Nln-Fw: 5'-CTGAAGGCCTGCTAAGCATC-3' and Nln-Rv: 5'-CGCATGGTTGTATTTTCCT-3').

Enzymatic activity

The enzymatic activity of Nln was determined in triplicate using a continuous assay with a quenched fluorescent substrate (QFS; 7-methoxycoumarin-4-acetyl-P-L-G-P-dK-(2,4-dinitrophenyl) as previously described (5). The Nln specific inhibitor Pro-Ile (5 mM) (37) was used to discern the peptidolytic activity attributed exclusively to Nln. The results were expressed as arbitrary units of fluorescence (UAF) per minute normalized by protein concentration.

Body composition

Body composition (free water, fat and lean mass) was estimated in 6 months old mice by dualenergy X-ray absorptiometry using a Hologic QDR 4500 scanner (Hologic, Waltham, MA) as described previously (38).

GTT, ITT and gluconeogenesis

Mice were fasted 12 hours and blood samples were taken before and at 15, 30, 60, 90, and 120 min after the injection of 2g/Kg glucose (glucose tolerance test, GTT) or 0.75U/Kg insulin (Insulin tolerance test, ITT). Gluconeogenesis was assessed by injecting 2g/kg sodium pyruvate after 16 hours fasting. In all three assays, glycemia was measured using a glucometer (Accu-Check Performa - ROCHE).

Western blot

For determining insulin signaling in vivo, we collected samples of muscle, liver and epididymal adipose tissue before and 5 min after tail vein injection of insulin (5 U/kg) as previously described (39). The samples were immediately stored in liquid nitrogen.

For western blot analyses, tissue lysates were homogenized in RIPA buffer containing inhibitors of proteases (Complete; ROCHE) and phosphatases (PhosSTOP; ROCHE). Samples were subjected to SDS-PAGE in a Bio-Rad electrophoresis apparatus (Mini-Protean). Proteins were transferred to a PVDF membrane (THERMO SCIENTIFIC PIERCE). After 2 hours in blocking buffer (Odyssey, LI-COR-Biosciences) the membranes were incubated with anti-Nln (Rabbit polyclonal antibody, ABCAM), anti-phosphoAKT (Ser 473), or anti-AKT (Rabbit polyclonal antibody, CELL SIGNALING) overnight at 4°C and then incubated with IRDye Secondary Antibody (Odyssey, LI-COR-BIOSCIENCES). The results were visualized in a Odyssey Infrared Imaging System (Odyssey, LI-COR- BIOSCIENCES) and was quantified by

densitometry with Odyssey Infrared Imaging software (Odyssey, LI-COR- BIOSCIENCES).

Histological analysis

We performed hematoxylin and eosin staining for histological analysis in gastrocnemius, soleus, liver and adipose tissue. For mitochondrial activity, we used a tetrazolium compound and 3,3' diaminobenzidine (DAB) to identified sites for succinate dehydrogenase (SDH) and cytochrome oxidase (COX), respectively, in fresh frozen sections of gastrocnemius, as previously described (40,41). All data acquisition and analysis were performed in a microscopy BZ-9000 (BIOREVE).

Real time PCR

We used real-time PCR (qPCR) to determine mRNA expression in liver, muscle and adipose tissue. Animals fasted for 12 hours were sacrificed by decapitation and tissues were removed, snap frozen in liquid nitrogen and stored in -80°C until use. Samples were homogenized and total RNA was isolated using Trizol protocol (TRIzol® Products Life Technologies). The total RNA was cleaned in RNeasy Mini Kit (QIAGEN) and the RNA integrity was assessed by electrophoresis on 1% agarose gels.

cDNA was synthesized from 1µg of total RNA with Moloney Murine Leukemia Virus Reverse Transcriptase (INVITROGEN) using random hexamer nucleotides. Standard curves for all primers were made to determine the amplification efficiencies of target and reference genes. Quantitative PCR was performed on an ABI Prism 7900 (APPLIED BIOSYSTEMS) sequence detection system with 100 nM primers and 20 ng of cDNA. Target mRNA expression was normalized to GAPDH expression and expressed as a relative value using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$) according to the manufacturer's instructions. Expression levels from genes of interest were normalized to WT control mice and presented as fold change. All primers sequences used herein are described in Table 1S.

Graded treadmill exercise test

Exercise capacity, estimated by total distance run, correlates with skeletal muscle work capacity. Exercise capacity was evaluated with a graded treadmill using protocols from previously published reports. Briefly, after being adapted to treadmill exercises over a week (10 min of exercise per session); mice were placed in the treadmill streak and allowed to acclimatize for at least 30 min. We used high and low intensity running regimens. High intensity running: Intensity of exercise was increased by 3m/min every 3 min until exhaustion. Exhaustion was defined as the time when animals were unable to keep pace with the treadmill for up to 1 min. Low intensity running: We used the average speed in the High intensity running and calculated 70% of this result (21m/min) to put all animals to run in this constant speed until exhaustion for maximum capacity of physical exercise.

Blood pressure

Measurement of blood pressure was performed as previously described (42). KO and WT were anesthetized with isoflurane and then a modified catheter (type MRE-025-in. outer diameter 0.012-in. inner diameter; Braintree Scientific, Braintree, MA, USA) was placed into the abdominal aorta via the femoral artery for measurement of arterial blood pressure. The blood pressure was measured using a transducer (MLT 1050 model) connected to a computer system for data analysis (PowerLab, AD Instruments, Colorado Springs, PA, USA) (43). To test blood pressure response to different drugs (neurotensin 1nM, bradykinin 5mg/kg and 10mg/kg and angiotensin II 100ng/kg) a second modified catheter was placed in the femoral vein.

Peptide extraction

Peptide extracts were prepared as previously described (44,45). Briefly, 6 month old mice male (n = 5) were sacrificed by decapitation, and gastrocnemius, soleus, liver and epididimal adipose tissue were collected and subjected to 10 s of microwave (1500 watts, ELECTROLUX) radiation in order to inactivate protein and peptide degradation. Each sample was homogenized (Polytron; BRINKMANN) in 10 ml of water, and incubated at 80°C for 20 min. After cooling on ice, 20 µL of 5 M HCl was added to give a final concentration of 10 mM and sonicated three times with 20 pulses (4 Hz). The homogenates were centrifuged at 1,500×g for 40 min at 4°C. After this point, the supernatants were collected and centrifuged at 15000×g for 40 min at 4°C. The resulting supernatants were collected and filtered through a Millipore membrane (MCWO 10000 – Amicon Ultra™, MILLIPORE). The flow-through was applied to C18-like Oasis columns (WATERS) (Figure 2). The peptide extracts were

resuspended in 100 μL of deionized water and kept at -80°C.

Peptide quantification

Peptide concentration was determined as previously described (44,46). The reaction was performed at pH 6.8 to ensure that only the amino groups of peptides and not those of free amino acids react with fluorescamine (47). Briefly, 2.5 μL of each sample was mixed with 25 μL of 0.2 M phosphate buffer (pH 6.8) and 12.5 μL of a 0.3 mg/ml fluorescamine solution in acetone. After vortexing for 1 min, 110 μL of water was added, and fluorescence was measured with a SpectraMax M2^{e} plate reader (MOLECULAR DEVICES) at an excitation wavelength of 370 nm and an emission wavelength of 480 nm. The peptide 5A (LTLRTKL), of known composition and concentration, was used as the standard reference for determining the peptide concentration.

Isotopic labeling

The isotopic labeling of peptides for semiquantitation was performed as originally described (48,49). Fifty micrograms of peptide extract from gastrocnemius, soleus, liver or epididymal adipose tissue were combined with 200 μL of 0.4 M phosphate buffer, pH 9.5. The pH was adjusted to 9.5 with 1 M NaOH, 6.4 μL of a solution 350 μg/μL D0 TMAB, D3-TMAB or D9-TMAB, dissolved in Me_2SO was added. After 10 min at room temperature, an appropriate volume of 1.0 M NaOH was added to the reaction mixture to adjust the pH back to 9.5, and the reaction was further incubated for 10 min. The addition of labeling reagent and alkaline solution was repeated six times over 2 h, and the mixture was incubated at room temperature for 30 min. After incubation, 30 μL of 2.5 M glycine was added to the reaction to quench any remaining labeling reagent. After 40 min at room temperature, the hydrogen and deuterium samples were combined and centrifuged at 800×g for 5 min at 4°C. The pH was adjusted to 9.0–9.5, and 3 μL of 2.0 M hydroxylamine was added to remove TMAB labels from Tyr residues. The addition of hydroxylamine was repeated twice more over 30 min. The samples were desalted with a C18 column (Oasis Millipore; Billerica, MA, USA). The peptides were applied to C18-like Oasis columns (WATERS), eluted with 100% methanol/015% trifluoracetic acid and dried in a vacuum centrifuge. Samples were resuspended in 10 μL of deionized water (Figure 2).

Mass spectrometry and MS and MS/MS data analyses

LC–MS/MS experiments were performed on a Synapt G2 QTOF mass spectrometer (WATERS) as described previously (44). To identify peptides, the raw data files were converted to a peak list format (mgf) by Mascot Distiller version 2.1.1 (MATRIX SCIENCE) and analyzed using the search engine MASCOT version 2.2 (MATRIX SCIENCE). The SwissProt database was used for searches with taxonomy *Mus musculus*, ''no enzyme'', and 0.2 Da of mass tolerance for MS and MS/MS precursor ions. Mascot searches were followed by manual interpretation to eliminate false positives. Quantification was performed by measuring the ratio of peak intensity for the various TMAB-labeled peptide pairs in the MS spectra. For this analysis, the monoisotopic peak and the peaks containing one or two atoms of ^{13}C were used. Multiple scans of the MS spectra were combined prior to quantitation (Figure 2).

Gene ontology analysis

The forty proteins obtained from the peptidomic analyses shown in Table 3S plus Nln (shown in Table 4S), were subjected to gene ontology (GO) clustering analysis using Biological Network Gene Ontology (BiNGO) software (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html) (50), a freely available plugin for the Cytoscape software, version 3.0.1 (http://www.cytoscape.org) (51). The selected ontology file was based on Biological Process, the annotation file was based on *Homo sapiens* databanks and the selected categories to be visualized discarded the evidence codes by overrepresentation after correction. The degree of functional enrichment for that list of proteins was obtained quantitatively (*p-value*) with the hypergeometric distribution statistical test (52,53), which worked independently inside of the BiNGO software application platform, , and the corrected *p-value* was obtained after applying FDR (Benjamini & Hochberg)correction with a significance level of 0.05. The selected reference test was the whole annotation from databases.

Construction of physical protein-protein interaction (PPPI) networks

To design and construct a potential physical protein-protein interaction (PPPI) network containing Nln and the proteins represented by peptides from the peptidomic analyses shown in Table 3S, we initiated data mining of human protein-protein interaction databases using them as seed list. This procedure was carried out inside the Cytoscape platform with the APID2NET Cytoscape Plug-in software (http://bioinfow.dep.usal.es/apid/apid2net.html) (54). The obtained network was named as Union (because it was originated by the union of proteins identified in the gastrocnemius+liver+soleus+adipose tissue samples), which was then submitted to GO analysis again, as above described.

Statistics

Values are expressed as means ± SEM. Statistical analyses were conducted by Student's unpaired t-test for independent samples or ANOVA followed by Bonferroni's test to compare more than two groups. P values < 0.05 were considered significant.

RESULTS

Gene-trap strategy for knockout of Nln expression in mice

The summarized strategy of generating Nln knockout mice (KO) by insertion of a gene-trap between exons 1 and 2 is illustrated in Figure 1A. The presence of the gene-trap in the Nln gene of the knockout mice was confirmed by genotyping (Figure 1B). To obtain a pure genetic background we bred F1 heterozygous Nln-deficient animals to the C57BL/6 mouse line for 10 consecutive generations. After 10 generations, the Nln mRNA, protein levels and enzymatic activity were investigated in various tissues, confirming the absence of Nln mRNA and protein expression in these KO mice (Figure 1C, D and E).

Initial phenotypical characterization of Nln knockout mice

Disruption of Nln expression caused neither embryonic lethality nor changes in the normal Mendelian distribution following intercrossing of the heterozygotes. KO mice could not be visually distinguished from wild type C57BL/6 litter-mates (WT) animals and had normal external appearance and fertility. KO and WT mice also showed similar muscle mass, free water and fat mass composition as analyzed by densitometry (Figure 3A, B and C). In contrast, KO mice showed a slight although statistically significant ($p<0.05$) ~10% decrease in body weight compared to WT (Figure 3D), however no difference in the specific mass of the internal organs was observed (Table 2S). There was also no obvious difference in the morphology of skeletal muscle, liver and epididymal adipose tissue stained with hematoxylin and eosin (H&E) (Figure 3E, F, G, H, I and J).

Insulin sensitivity and gluconeogenesis tests

The basal glucose levels of WT and KO are similar (Figure 4A). However, the intraperitoneal glucose tolerance test (GTT) done in WT and KO fasted for 12 hours shows lower glucose concentrations at 15, 30, 60 and 90 min in KO compared to WT (Figure 4B and C). To further assess insulin sensitivity we performed the insulin tolerance test (ITT) in 12 hours fasted mice. Considering the normalized curve for the ITT data (Figure 4D) and the constant rate for glucose disappearance (K_{ITT} - Figure 4E), KO showed an increase of insulin sensitivity when compared to WT. The liver gluconeogenesis capacity of KO is higher compared to WT animals (Figure 5A and B), as shown by the intraperitoneal pyruvate tolerance test in animals fasted for 16 hours.

Comparative analyses of several regulatory genes involved in liver gluconeogenesis were performed in WT and KO using quantitative PCR (qPCR). These results suggest that KO have increased liver mRNA for the fructose bisphosphatase 1 (*Fbp1*), peroxisome proliferator-activated receptor gamma (*PPARγ*) and cAMP responsive element binding protein 1 (*Creb1*) (*Cox4i1*), which is in agreement with an increased liver gluconeogenesis of KO when compared to the WT animals (Figure 5C).

Graded treadmill exercise tests

WT and KO mice performance on a treadmill was evaluated in two different tests. First, animals were subjected to a high intensity running regime that increased the speed stepwise every 3 min up to the ratio of 3m/min, until exhaustion. These results showed no statistical difference between WT and KO animals (Figure 6A and B); on average, WT and KO mice ran 422 m and 337m, respectively, until complete exhaustion. Second, animals were subjected to a low intensity running regime (21m/min constant) until exhaustion and the KO (637m) ran

significantly (~36%) less when compared to WT (997m) (Figure 6C and D), suggesting that Nln is linked to oxidative metabolic capacity and physical exercise endurance.

Molecular basis of increased glucose metabolism in KO mice

To further investigate the molecular basis correlated with the higher insulin responsiveness in KO, we analyzed the insulin signal transduction pathway through AKT^{Ser473} phosphorylation in the gastrocnemius, liver and epididymal adipose tissues of both WT and KO animals. Whereas the basal levels of AKT^{Ser473} phosphorylation were similar between WT and KO animals (Figure 7A, B and C), following a stimulus with an acute intravenous insulin injection it was higher in gastrocnemius (Figure 7A) and epididymal adipose tissue (Figure 7B) but not in the liver (Figure 7C) of KO compared to WT animals.

Fast twitch type II fibers remodeling in Nln knockout mice in gastrocnemius

The enzymatic activity of Nln in WT gastrocnemius (predominantly fast twitch type II fibers) is approximately three times lower than in the soleus (predominantly slow twitch type I fibers), liver or epididymal adipose tissues (Figure 8). Based on this finding and since KO mice ran less than WT mice in the low intensity running regime until exhaustion, we decided to investigate a possible remodeling of fiber compositions in skeletal muscle. Using qPCR, we analysed mRNA expression of specific genes for slow twitch type I (Tnni1, Tnnt1, Tnnc1 and Myh7) and fast twitch type II fibers (Tnni2, Pvalb, Myh1, Myh2 and Myh4) in gastrocnemius and soleus (Figure 9A and B). KO showed a decrease in mRNA expression typical for slow twitch type I fibers in gastrocnemius but not in soleus, while no differences were observed for fast twitch type II fibers in both skeletal muscles (Figure 9A and B).

Next, histological sections of gastrocnemius were stained for the mitochondrial enzymes succinate dehydrogenase (SDH) (Figure 10A and B) and cytochrome oxidase (COX) (Figure 10D and E). Indeed, KO showed a significant decrease in the number of oxidative fibers (slow twitch type I) stained for both SDH (Figure 10C) and COX (Figure 10F) in the gastrocnemius tissue.

Distinctive intracellular peptide profile of KO and WT in skeletal muscle, liver and adipose tissue

Semi-quantitative peptidomic analyses using isotopic labeling and LC-ESI-MS were performed to identified possible differences in the global peptide profile between WT and KO animals (Table 3S). Altogether, 736 MS peptide spectra were quantified including those peptides that were not sequenced by MS/MS (Table 3S). All of these peptides were fragments of intracellular proteins, of which the relative levels seemed slightly affected by the lack of Nln. In accordance to previous reports (36,44,49), we postulated that Nln should be a key enzyme to metabolize peptides that have increased or decreased two fold (more than 100% of increase or more than 50% of decrease) in KO compared to WT mice; peptides that increase are considered potential Nln substrates, while peptides that decreased are considered potential Nln products. Thus, from the total of peptides identified (Table 3S) only a small number could be considered either substrates or products of Nln (Table 1). In the gastrocnemius two peptides derived from troponin I, fast skeletal muscle, SADAMLKALLGSKHK and DMEVKVQKSSKELEDMNQKL, increased more than 2 fold (Table 3SB). In the adipose tissue two different peptides, one derived from the acyl-CoA-binding protein (VEKVDELKKKYGI) and one derived from the hemoglobin subunit alpha (LASVSTVLTSKYR) increased more than 2 fold (Table 3SA). In the liver, several endocannabinoid peptides containing the mouse hemopressin (PVNFKLLSH) endocannabinoid signature (SDLHAHKLRVDPVNFK, RVDPVNFKLLS, VDPVNFKLL, VDPVNFKLLSH and RVDPVNF) or the previously identified antinociceptive AGH peptide (AGHLDDLPGALSA) decreased more than 2 fold (Table 3SC). In the soleus, only the peptide FASFPTTK derived from the hemoglobin alpha subunit decreased more than 2 fold (Table 3SD). None of the peptides identified in the gastrocnemius or in the epididymal adipose tissue decreased more than 2 fold. In liver and soleus tissues none of the identified peptides increased more than 2 fold. Moreover, all the peptides identified here to change the relative concentration among KO and WT were in the size range of Nln substrate specificity, which are peptides containing from ~5-20 amino acids. These data suggests a great specificity of Nln for metabolizing intracellular peptides and/or a compensatory mechanism occurring in the KO mice.

Nln knockout mice did not show any difference in blood pressure parameters

A possible key role of Nln in degrading circulating bioactive peptides was first investigated through blood pressure monitoring, introducing a catheter in the femoral artery as previously described (42). Under basal conditions, WT and KO have similar mean arterial pressure (109.0 ±1.5 *vs* 108.0 ±1.5 mmHg, respectively; Figure 11A) and heart rate (668.4 ±8.8 *vs* 652.0 ±14.5 bpm, respectively; Figure 11B). Next, we challenged the participation of Nln in blood pressure regulation injecting vasoactive peptides such as bradykinin, neurotensin, and angiotensin II in two different doses (5 and 10 mg/kg). However, no significant differences were seen in the blood pressure effects of these peptides comparing WT to KO responses (Figure 11C, D and E). These data suggest that Nln alone is not a key enzyme in blood pressure regulation.

Gene ontology analysis and physical protein-protein interaction (PPPI) networks

The intracellular peptides identified by LC-ESI-MS in the four peripheral tissues (adipose tissue, gastrocnemius, liver and soleus) and shown in Table 3S were used to generate a list of thirty-nine proteins, which together with Nln itself were used for further *in silico* analysis (Table 4S). To discover biological processes affected by the ablation of Nln, these proteins were submitted to gene ontology (GO) analyses, (Table 2) considering significant results only when the p-value was below 10^{-5}. The list of biological process and genes related to these peptides are shown in Table 2. Most of the genes identified in the GO analyses are involved in basal energetic metabolism, such as glycolysis and glucose metabolism (ALDOA, G3PT, PGAM2, CPSM) and muscle contraction (ALDOA, PGAM1, PGAM2, SMPX, MYL1). qPCR analyses were performed to evaluate the effect of Nln KO in the expression of several of these genes, in the gastrocnemius and soleus (Figure 12). These data suggest that Nln KO enhances CPSM and reduces PGAM1 and 2 gene expression in the gastrocnemius, which is not observed in the soleus (Figure 12).

Moreover, in order to identify other biological processes in which Nln might act as a direct or indirect regulator, we started data mining in physical protein-protein interaction databases by using as input the forty proteins from Table 4S and constructed a network named "union network" (Figure 1S), because of the union of non-overlapping proteins found in the gastrocnemius, liver, soleus, and adipose tissue samples, together with Nln. Despite a few proteins were not found in the human PPPI databases (used here because it is more complete and updated than the mouse database), the resulting network presented 229 proteins (nodes) and 680 connections, between the forty input proteins and other related ones acting as substrates or interacting partners. In this complex network Nln is initially not part of the biggest network (Figure 1S). However, as suggested by our results, Nln can interfere with other proteins, pathways and biological processes. Thus, the obtained *union network* was submitted to a new GO analysis, for enlarging the list of genes and biological processes that could be affected by the knockout of Nln (Table 5S). Besides the general and tissue-specific biological processes already found in Table 2, other biological processes related to the nervous system were also identified, such as: a) regulation of nerve impulse transmission through synapses; b) nervous system development; c) learning and memory; d) regulation of synaptic plasticity; e) cell morphogenesis involving neuron differentiation; f) response to different types of stressor agents; g) DNA damage and repair pathways; h) regulation of cell proliferation (S- and M- phases); i) intracellular signaling; and j) protein stability, complex assembly and post-translation modifications. Therefore, *in silico* analysis using a system biology approach (55), highlights Nln as a (indirect) regulator of several physiological and cellular processes in addition to energy metabolism. Further biological investigation is necessary to further validate these *in silico* data.

DISCUSSION

The major findings presented here are the generation and phenotype characterization of Nln knockout mice (KO). Nln was first described as a novel neurotensin-cleaving enzyme and received the first denomination of 'neurotensin-degrading neutral metalloendopeptidase' (3). Whereas pharmacological studies have associated Nln to distinct physiological functions and several pathologies, one can reasonably argue that its actual physiological role is still elusive (56). Here, the characterization of Nln KO mice uncovered the physiological relevance for this oligopeptidase in the regulation of energy metabolism. Insulin

resistance and diabetes type 2 are for a long time associated with increased cardiovascular risk factors, including dyslipidemia, hypertension, impaired fibrinolysis, coagulation and obesity (57). Together with cardiovascular diseases, diabetes type 2 is one of the leading causes of mortality and mobility in the modern world (35). KO mice were shown here to have increased glucose tolerance, insulin sensitivity and liver gluconeogenesis. Hence, these data suggests a novel therapeutic possibility, which is to inhibit Nln for improving glucose uptake and peripheral insulin sensitivity.

Glucose metabolism is vital for health as it is an important fuel for physiological functions (58). Insulin is secreted by pancreatic β-cells and regulates the metabolism of glucose after food intake, increasing glucose uptake by skeletal muscle and adipose tissue and decreasing hepatic glucose production by blocking glycogenolysis and gluconeogenesis (59). The most important effect of insulin is the increase of glucose uptake, which can be mediated through AKT phosphorylation which induces the translocation of GLUT4 to the plasma membrane (60-62). KO mice have increased glucose tolerance, insulin sensitivity and liver gluconeogenesis. Part of these results is related to an increase in AKT phosphorylation in skeletal muscle and adipose tissues of KO in response to insulin stimulation. The lack of changes in liver AKT phosphorylation after insulin administration can be explained the similar basal glycemia of KO and WT animals. KO mice exhibit more gluconeogenesis than WT animals, which is in accordance with the increased mRNA expression of specific genes that code for enzymes involved in gluconeogenesis in the liver (63-66). Moreover, *in silico* and experimental analyses corroborates that Nln is linked to energy metabolism and homeostasis regulation.

Accordingly, KO mice show alteration in mRNA expression of genes typical for slow twitch type I fibers (reduction of Tnni1, Tnnic1, Tnnt1 and Myh7), urea cycle (increase of CPSM) and glycolytic pathway (reduction of PGAM1/2) in the gastrocnemius but not in the soleus. Moreover, the histological analyses of SDH and COX in the gastrocnemius suggested a reduced mitochondrial oxidative activity. Several reports have shown that the skeletal muscle fiber composition is an important determinant in metabolism. Moreover, it is well established that slow twitch type I fibers act in glucose and lipid metabolism and endurance training promotes an increase of type I fiber muscles (67-72). Indeed, the KO mice ran less than WT mice in the low intensity running regime until exhaustion. Taking altogether, these results further suggest that Nln is linked to oxidative metabolic capacity.

Several previous reports correlated metabolic improvement with a lack of peptidase activity, suggesting that peptide metabolism plays an important function in homeostasis regulation (29,30,33). The phenotypic changes observed in the angiotensin-converting enzyme (ACE) transgenic mice are not affected by losartan, an antagonist of the angiotensin II-receptor, suggesting that the renin-angiotensin system was likely not responsible for this phenotype (35). In contrast, Nln activity is significantly lower in the fat tissue of mice that possessed three copies of the angiotensin-converting enzyme (ACE) gene. A possible explanation for the latter observation is that ACE acts as a dipeptidyl-carboxypeptidase releasing dipeptides, which have been shown to specifically inhibit Nln (35). Reduced Nln activity in the fat tissue of ACE transgenic mice was associated to the distinctive intracellular peptide profile of these ACE transgenic mice. These data let us propose that intracellular peptides could improve insulin sensitivity in the adipose tissue of ACE transgenic mice (35). Further investigation shown that the levels of specific intracellular peptides are increased in the adipose tissue of Wistar rats that were fed a high-caloric Western diet, compared with rats fed a control diet. Two of these intracellular peptides (LDBI, GDVNTDRPGLLDL and DBI, TVGDVNTDRPGLLDL) were reintroduced into 3T3-L1 adipocytes (treated or not with palmitate to induce insulin resistance) and shown to increase insulin-stimulated glucose uptake (36). These peptides were also shown to interact with specific proteins only in the adipose tissue from rats fed a high-caloric Western diet (36). Therefore, suggesting that intracellular peptides could be endogenously involved in insulin-stimulated glucose uptake.

Previous biochemical, structural and pharmacological evidences supports the high specificity of Nln for degrading small bioactive peptide substrates (9-11). Here, semi-quantitative mass spectrometry analysis shows that specific intracellular peptides were affected in the KO compared to the WT mice. Therefore, it is likely that the phenotypes observed in the KO should be mediated by the lack of Nln enzymatic activity, and as a consequence a shift in the intracellular

peptide profile. A possible molecular mechanism related to intracellular peptide function includes binding to a protein, and as a consequence modification of the ability of this protein to interact with other proteins. It is important to mention that intracellular peptides, distinctly from classical biologically active peptides, are suggested to function downstream of plasma membrane receptors, regulating signal transduction pathways from inside the cells (36,47,73,74). Synthetic peptides have been extensively used to disrupt intracellular protein-protein interactions and alter protein functions. In some cases, synthetic peptides activate a protein by mimicking the effect of a protein-protein interaction; in other cases the peptides inhibit the protein by blocking a protein-protein interaction. Another possibility includes the binding of the peptide to a protein followed by alteration of its folding. Many proteins undergo conformational changes after binding to a peptide (75). It is possible that endogenous peptides perform similar functions as found for synthetic peptides (74). Indeed, genetic analysis of *Caenorhabditis elegans* shows that under stress of mitochondrial protein misfolding, peptide signals are emitted from the mitochondrial matrix to the nucleus, which regulate nuclear-encoded mitochondrial chaperone genes (76). Moreover, thimet oligopeptidase inhibition using siRNA has been shown to affect beta-adrenergic signal transduction in parallel to the intracellular peptide profile (73). Therefore, it is an exciting possibility to suggest that the phenotype observed here in the KO mice, are mediated by intracellular peptides due to an oligopeptidase (77) action.

Nln have been suggested to participate in several important physiological processes like cardiovascular and renal homeostasis, pain perception, regulation of the reproductive axis and recently also in the brain response to stroke (26,28,78-81). Also many studies using shRNA or chemical inhibitors supported the involvement of Nln in these physiological processes (28,78,82). Even though Nln is expressed widely in the central nervous system and in peripheral organs of mammals (13,83,84), its absence, as shown here, is not lethal. KO mice reproduce normally, and have normal blood pressure and heart rate when compared to WT mice. It has been previously suggested that *in vivo* Nln contributes to the metabolism of several endogenous peptides including neurotensin, bradykinin, angiotensins, substance P, opioids (i.e. metorphamide, dynorphin A_{1-8}), somatostatin and hemopressin (5,10,26,80). The presence of Nln activity in vascular endothelial cells suggested a possible physiological role for Nln in blood pressure control (79). However, even after neuropeptide injection (bradykinin, angiotensin II and neurotensin) KO and WT mice have similar behaviors in blood pressure alterations. A parallel study conducted in our laboratory suggested that Nln knockout only affects the levels of Met-enkephalin and octapeptide, thus, exerts only a minor effect on intracellular peptide metabolism in the brain (85). Altogether, these data suggests that other peptidases may efficiently compensate the lack of Nln in physiological neuropeptide metabolism. However, further investigations are necessary to uncover the enzyme(s) involved in such compensatory mechanisms.

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FOOTNOTES

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[2]The abreviations used are: Creb1, cAMP responsive element binding protein 1; Fbp1, fructose bisphosphatase 1; FoxO1, forkhead box O1; FoxO3, forkhead box O3; FoxO4, forkhead box O4; G6Pase, glucose-6-phosphatase, catalytic; Gck, glucokinase; Pck, phosphoenolpyruvate carboxykinase, cytosolic; Cox5i1, cytochrome c oxidase subunit IV isoform 1; PPARα, peroxisome proliferator activated receptor alpha; PPARγ, peroxisome proliferator activator receptor gamma; CS, citrate synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nln, neurolysin; AKT, protein kinase AKT; DPP-4, dipeptidyl peptidase-4; ACE, angiotensin-converting enzyme; Tnni1, troponin I, skeletal, slow 1; Tnnc1, troponin C, cardiac/slow skeletal; Tnnt1, troponin T1, skeletal, slow; Tnni1, troponin I, skeletal, fast 2; Myh1, myosin, heavy polypeptide 1, skeletal muscle, adult; Myh2, myosin, heavy polypeptide 2, skeletal muscle, adult; Myh4, myosin, heavy polypeptide 4, skeletal muscle; Myh7, myosin, heavy polypeptide 7, cardiac muscle, beta; MYO, myostatin; PARV, parvin alpha; SDH, succinate dehydrogenase; COX, cytochrome oxidase; PGAM1, phosphoglycerate mutase 1; PGAM2, phosphoglycerate mutase 2; CPSM, carbamoyl-phosphate synthase 1, mitochondrial; APOA2, apolipoprotein A-II; MYL1, myosin, light chain 1; SMPX, small muscle protein X-linked; ALDOA, aldolase A, fructose-bisphosphate; GTT, glucose tolerance test; ITT, insulin tolerance test; WT, wild type; HET, heterozygous; KO, knockout; AUC, area under the curve.

FIGURE LEGENDS

FIGURE 1. Generation of Nln knockout mice. A, representation of Nln gene structure and the location of the NPX481 line gene-trap insertion. The position of the Nln *locus* on a schematic representation of the mouse chromosome 13 is shown as a red vertical line (chr13-qD1). The Nln exons are represented as blocks connected by arrowed lines, where blue blocks indicate the coding and black blocks the untranslated regions. B, PCR for genotyping of Nln-knockout animals. C, qPCR showing absence of gene expression in KO animals in different tissues (ND, not detected). D, Western blot showing Nln protein expression (arrow) in different organs (kidney, liver and brain) in WT and KO. E, enzyme activity confirming the results of the genotyping and qPCR (WT, wild-type; HET, heterozygous; KO, Nln knockout).

FIGURE 2. Scheme for intracellular peptide extraction, labeling and LC-MS/MS analysis. First, the scheme shows briefly step by step the protocol for intracellular peptide extraction. For isotopic labeling with TMAB, we used three isotopic tags for each replicate. Intracellular peptides extracted from adipose tissue, gastrocnemius, liver and soleus were labeled with D0-TMAB, D3-TMAB and D9-TMAB and pooled as indicated in three different runs. The runs were labeled with reverse isotopic tags as control of the reactivity. The relative quantification is performed by measuring the intensity of the peak of peptide labeling with each isotopic tag from MS, taking an average of these values and

comparing the ratio of KO to the WT. For peptide identification the MS/MS spectra were analyzed with a Mascot program to identify the sequences followed by manual interpretation to eliminate false positives.

FIGURE 3. Body weight, basal glucose levels and body composition. A, B and C, body composition of muscle mass (A) free water (B) and fat (C) (n = 6). D, average body weight of WT and 24-weeks-old KO animals (n = 6). Data are presented as means ± SEM. Student's unpaired t-test: * $p < 0.05$. Histological analysis: E and B, muscle histological sections (gastrocnemius) stained with hematoxylin and eosin (H&E) of 24-weeks-old WT and KO animals, respectively. G and H, liver histological sections stained with H&E of 24-weeks-old WT and KO animals, respectively. I and J, epididymal adipose tissue histological sections stained with H&E of 24-weeks-old WT and KO animals, respectively (n=5).

FIGURE 4. Glucose and insulin tolerance test. A, basal blood glucose levels after fasting for 12 hours (n = 6). B, glucose tolerance test (GTT) after fasting for 12 hours of 24-weeks-old WT and KO animals (n = 6). C, area under the curve (AUC) for the glucose tolerance test. D, insulin tolerance test (ITT) after fasting for 12 hours of 24-weeks-old WT and KO animals (n = 6). E, calculation of the constant of glucose disappearance (KITT) for the insulin tolerance test. Data are presented as means ± SEM. Student's unpaired t-test or ANOVA followed by Bonferroni's test:. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIGURE 5. Pyruvate tolerance test and hepatic gluconeogenesis regulatory enzymes. A, pyruvate tolerance test of 24-weeks-old WT and KO animals to check the level of gluconeogenesis in the liver (n = 6). B, area under the curve for the pyruvate tolerance test. C, quantitative real time PCR for genes that are related to gluconeogenesis in the liver (n = 6). Data are presented as means ± SEM. Student's unpaired t-test or ANOVA followed by Bonferroni's test:. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIGURE 6. Resistance and endurance running test. A, running performance with high intensity individual and group average of 24-weeks-old WT and KO animals (n = 7). B, running time average of the high intensity test. C, running performance with low intensity individual and group average of 24-weeks-old WT and KO animals (n = 7). D, running time average of the low intensity test. Data are presented as means ± SEM. Student's unpaired t-test: ** $p < 0.01$.

FIGURE 7. AKT glucose signaling analysis by western blotting. A, B and C, Western blot showing AKT phosphorylation without (-) and with (+) insulin stimulation normalized to GAPDH in 24-weeks-old WT and KO animals (n = 6). Levels of AKT phosphorylation in each tissue is represented by the ratio phosphorylated (pAKT) (Ser473) to total (t-AKT). Data are presented as means ± SEM. ANOVA followed by Bonferroni's test:. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIGURE 8. Nln enzymatic activity in skeletal muscle, adipose tissue and liver. Soleus (predominantly slow twitch type I fibers), gastrocnemius (predominantly fast twitch type II fibers), liver and epididymal adipose tissue of C57BL/6 (n=3). Data are presented as means ± SEM. ANOVA followed by Bonferroni's test:. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIGURE 9. Real time PCR analysis of type I/II specific gene and myosin heavy chain gene expression in different skeletal muscle. A, quantitative real time PCR for specific genes from slow twitch type I fibers and fast twitch type II fibers in gastrocnemius (n = 6). B, quantitative real time PCR for specific genes from slow twitch type I fibers and fast twitch type II fibers in soleus (n = 6). Data are presented as means ± SEM. Student's unpaired t-test: ** $p < 0.01$ or *** $p < 0.001$.

FIGURE 10. Mitochondrial enzymatic activity in gastrocnemius. A and B, muscle histological sections showing the succinate dehydrogenase (SDH) staining of WT and KO animals of 24-weeks-old, respectively. C, relative quantification of histochemical succinate dehydrogenase staining (n = 4).

D and E, muscle histological sections showing cytochrome oxidase (COX) staining of WT and KO mice 24-weeks-old, respectively. F, relative quantification of histochemical cytochrome oxidase (n=4). Note, stronger staining fibers are more oxidative (slow twitch type I) and light staining fibers are more glycolytic (fast twitch type II). Data are presented as means ± SEM. Student't unpaired t-test:. * $p < 0.05$.

FIGURE 11. Blood pressure. A and B, mean arterial pressure (MAP) (expressed in mm Hg) and heart rate (expressed in beats per minute BPM) measured by femoral artery catheterization compared KO and WT respectively (n=12). C, effect of bradykinin on MAP after bradykinin injection in femoral vein (5 or 10 mg/kg) (n=7). D, effect of neurotensin on MAP after neurotensin injection in femoral vein (1nM/mice) (n=8). E, time course of the effect of angiotensin II on MAP after angiotensin II injection in femoral vein (100ng/kg) (n=8). Data are presented as means ± SEM. Student's unpaired t-test or ANOVA followed by Bonferroni's test shows no different statistical significance between groups.

Figure 12. qPCR for genes obtained after Gene Ontology (GO) analyses. qPCR in either WT (white bars) or KO (black bars) animals, both 24-weeks-old (n = 4). Several additional genes identified after Gene Ontology (GO) to be related to muscle contraction,metabolic and glucose metabolic process analyses were evaluated. **Upper panel**, gastrocnemius; **lower panel**, soleus. Data are represented as means ± SEM. Student's unpaired t-test: * $p < 0.05$; ** $p < 0.01$.

TABLE

Table 1 Summary of intracellular peptides quantification by LC-ESI MS

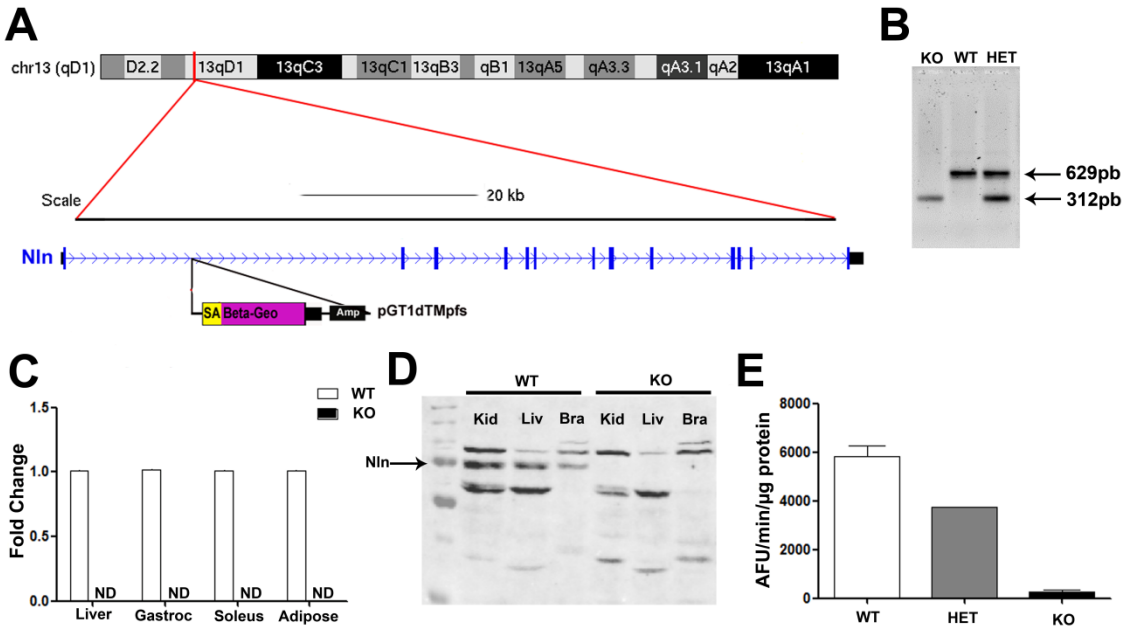
	MS Spectra	≥ 2.0 Increased	≤ 0.5 Decreased	≤ 0.5 and ≥ 2.0 not changed
Gastrocnemius	318	19 (6%)	0 (0%)	299 (94%)
Soleus	112	2 (2%)	8 (7%)	102 (91%)
Liver	189	2 (1%)	22 (12%)	165 (87%)
Adipose	110	15 (14%)	0 (0%)	95 (86%)

Footnote: Global analysis of intracellular peptides profile in soleus (predominantly slow twitch type I fibers), gastrocnemius (predominantly fast twitch type II fibers), liver and epididymal adipose tissue quantified by LC-ESI MS as Nln substrate (ratio KO/WT≥ 2. 0), product (ratio KO/WT≤ 0.5) or neither substrate or product (ratio KO/WT 0.5 and ≤ 2.0). Percentage means the amount of intracellular peptides that are substrates, products or not cleaved by Nln (n=5).

Table 2. Gene Ontology (GO) obtained for the 40 proteins represented by the peptides identified in the gastrocnemius, soleus, liver and adipose tissues, including the Nln

GO-ID	p-value	Biological Process Description	Genes in test set
6941	4,8E-20	striated muscle contraction	ALDOA |PGAM2 |TNNI2 |SMPX |MYL1 |MYG |TNNT3
6936	4,8E-12	muscle contraction	ALDOA |PGAM2 |TNNI2 |SMPX |MYL1 |MYG |TNNT3
3012	1E-11	muscle system process	ALDOA |PGAM2 |TNNI2 |SMPX |MYL1 |MYG |TNNT3
3009	1,1E-08	skeletal muscle contraction	TNNI2 |MYG |TNNT3
50881	1,2E-07	musculoskeletal movement	TNNI2 |MYG |TNNT3
50879	1,2E-07	multicellular organismal movement	TNNI2 |MYG |TNNT3
15671	2,9E-06	oxygen transport	HBB |HBA
46461	9,6E-06	neutral lipid catabolic process	APOA2 |CPSM
44269	9,6E-06	glycerol ether catabolic process	APOA2 |CPSM
46464	9,6E-06	acylglycerol catabolic process	APOA2 |CPSM
15669	1,9E-05	gas transport	HBB |HBA
6110	2,1E-05	regulation of glycolysis	PGAM1 |G3PT
46503	2,1E-05	glycerolipid catabolic process	APOA2 |CPSM
6006	2,1E-05	glucose metabolic process	ALDOA |PGAM2 |CPSM |G3PT
43471	6,8E-05	regulation of cellular carbohydrate catabolic proc	PGAM1 |G3PT
43470	6,8E-05	regulation of carbohydrate catabolic process	PGAM1 |G3PT
44275	9,5E-05	cellular carbohydrate catabolic process	ALDOA |PGAM2 |CPSM
19318	9,5E-05	hexose metabolic process	ALDOA |PGAM2 |CPSM |G3PT

Figure 1



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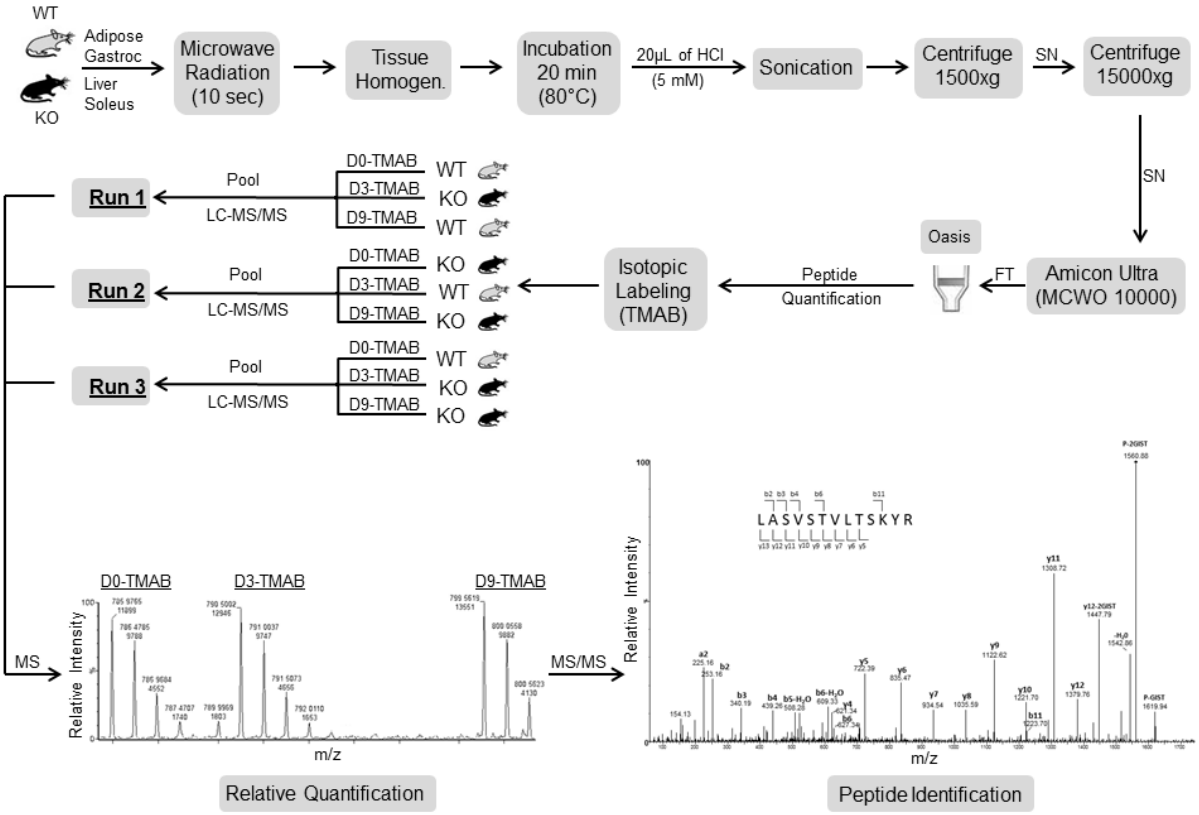

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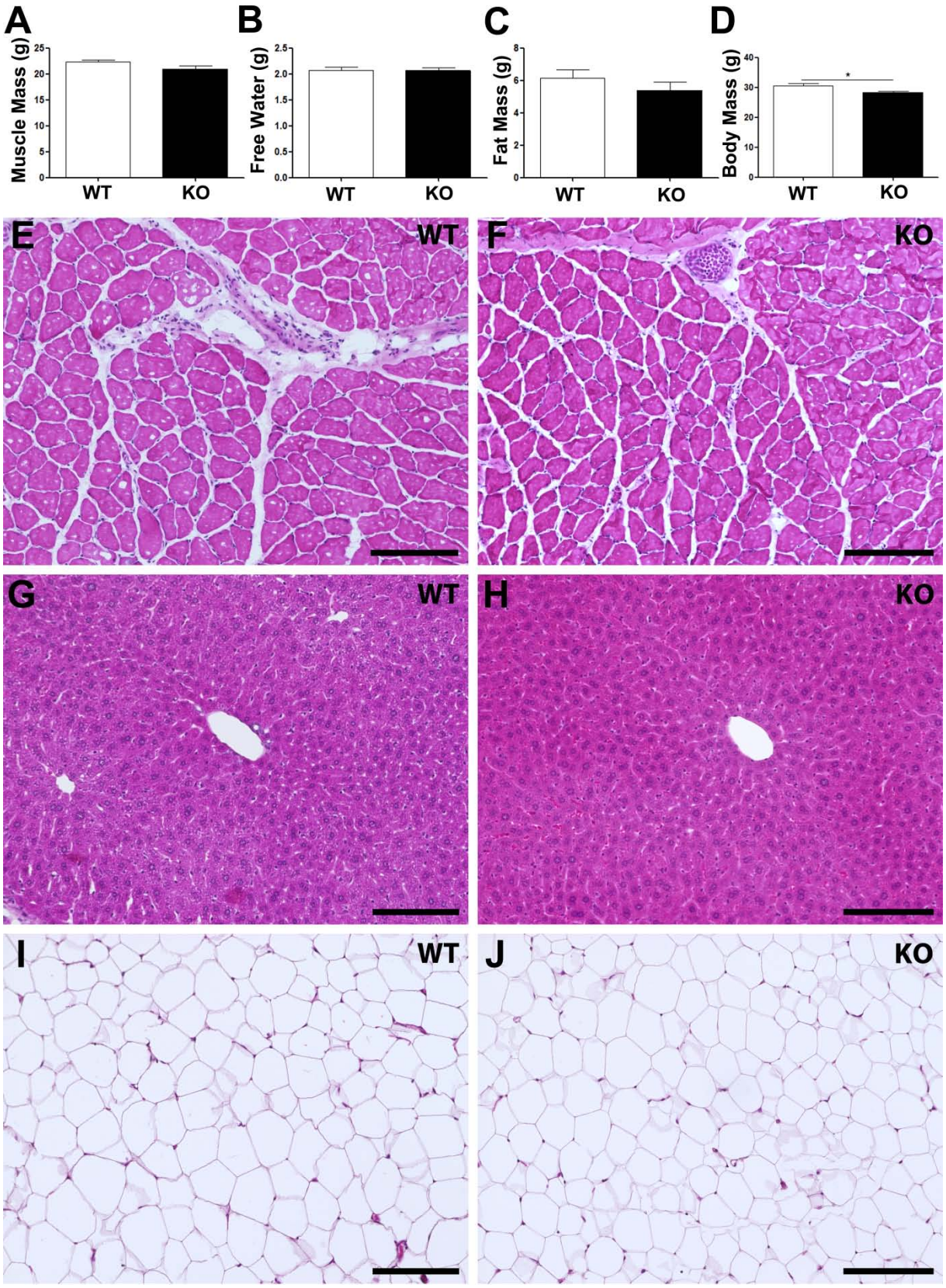

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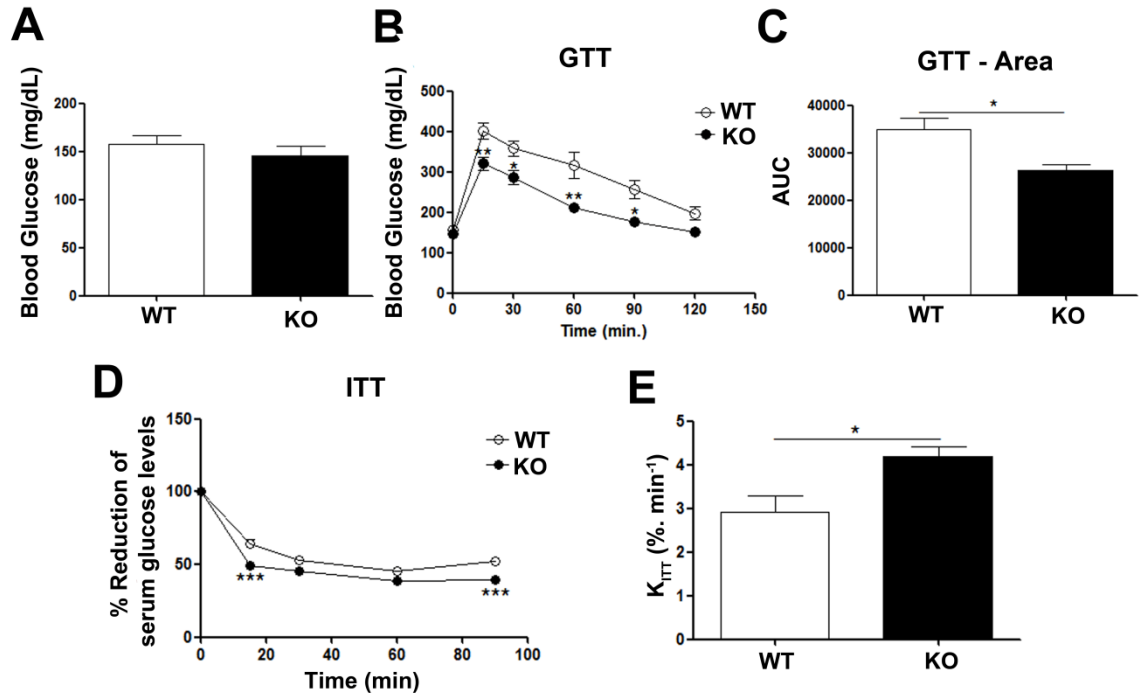

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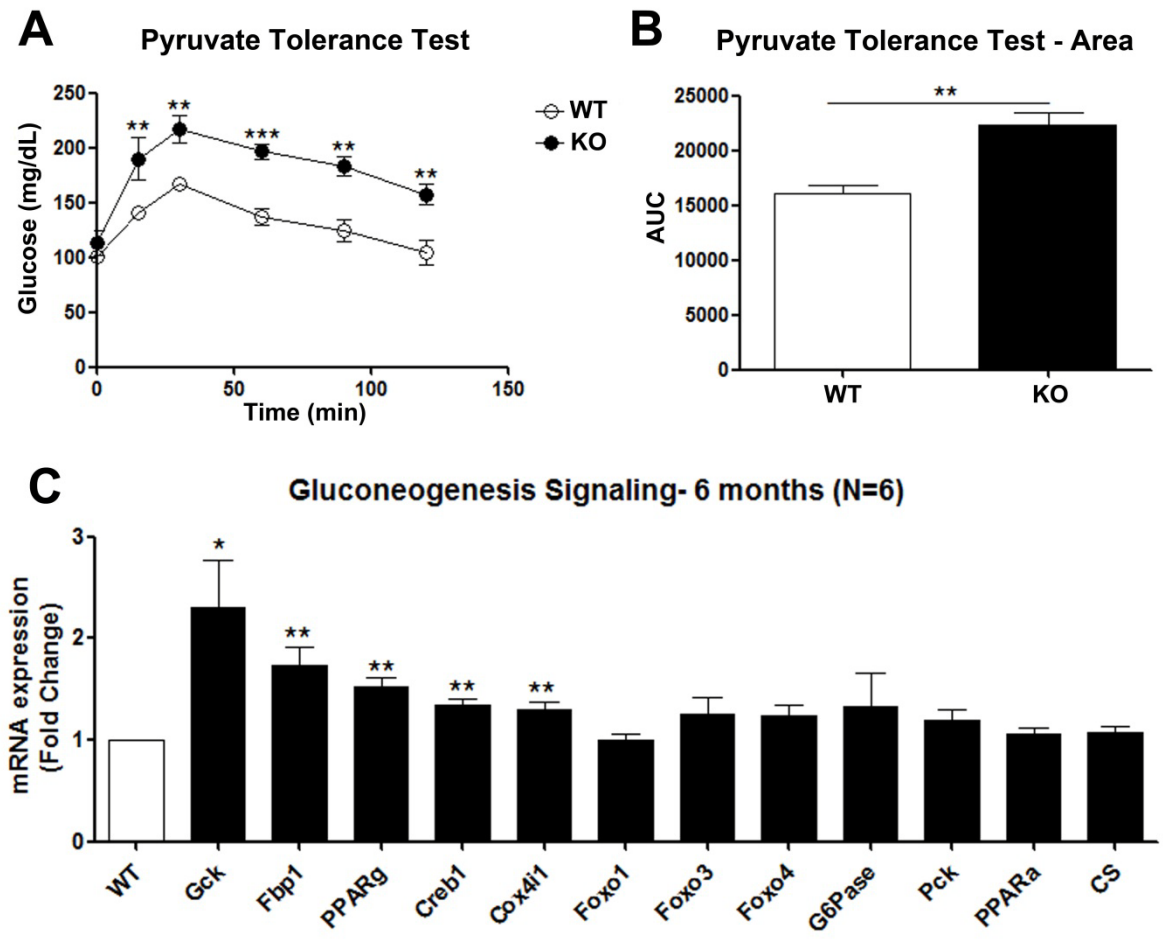

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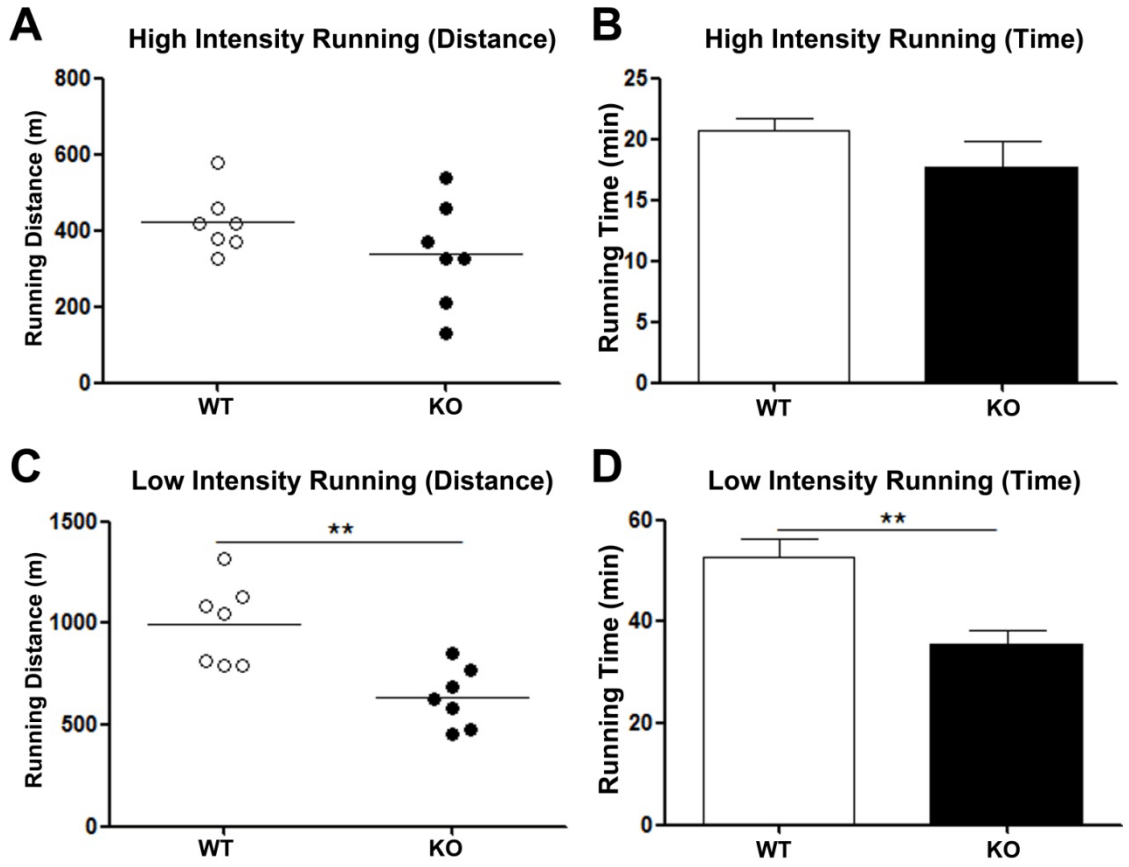

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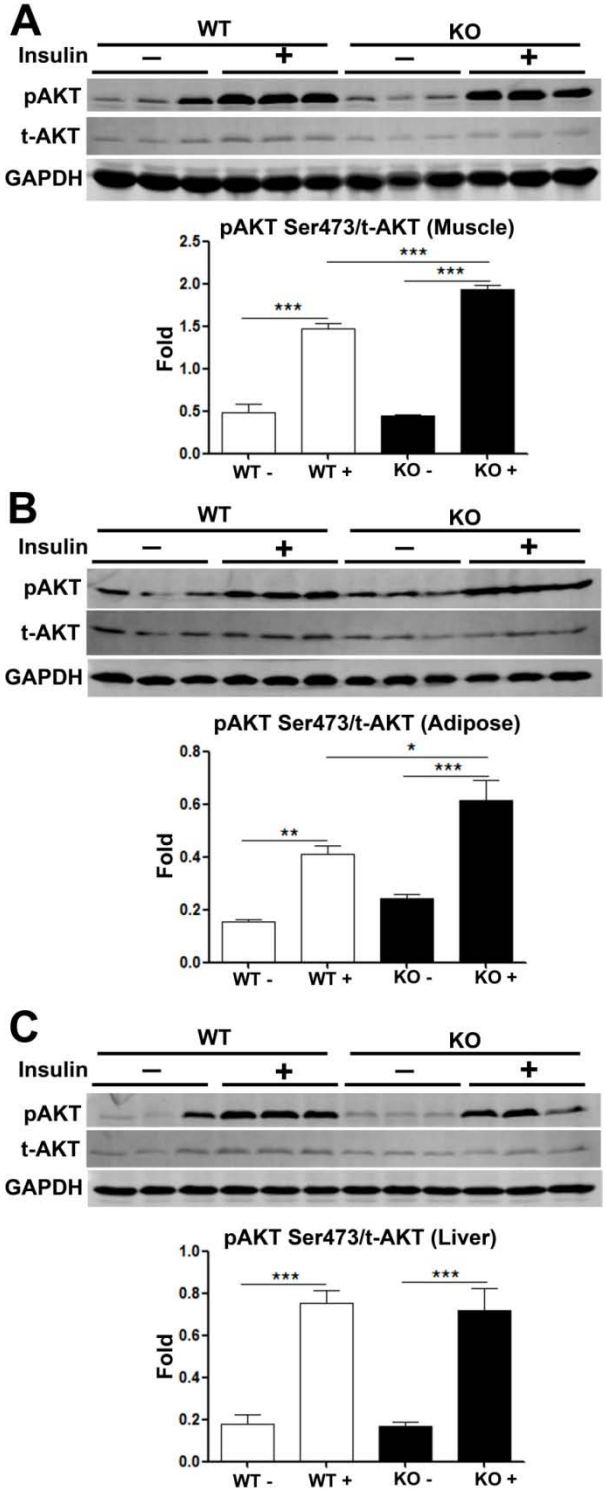

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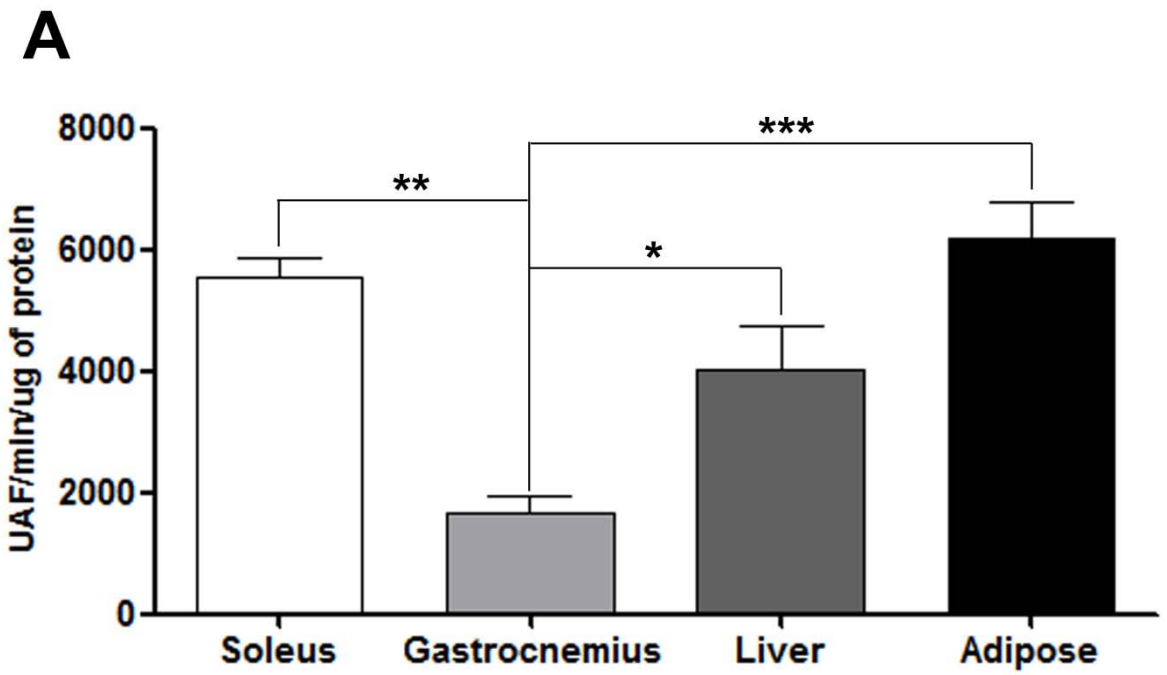

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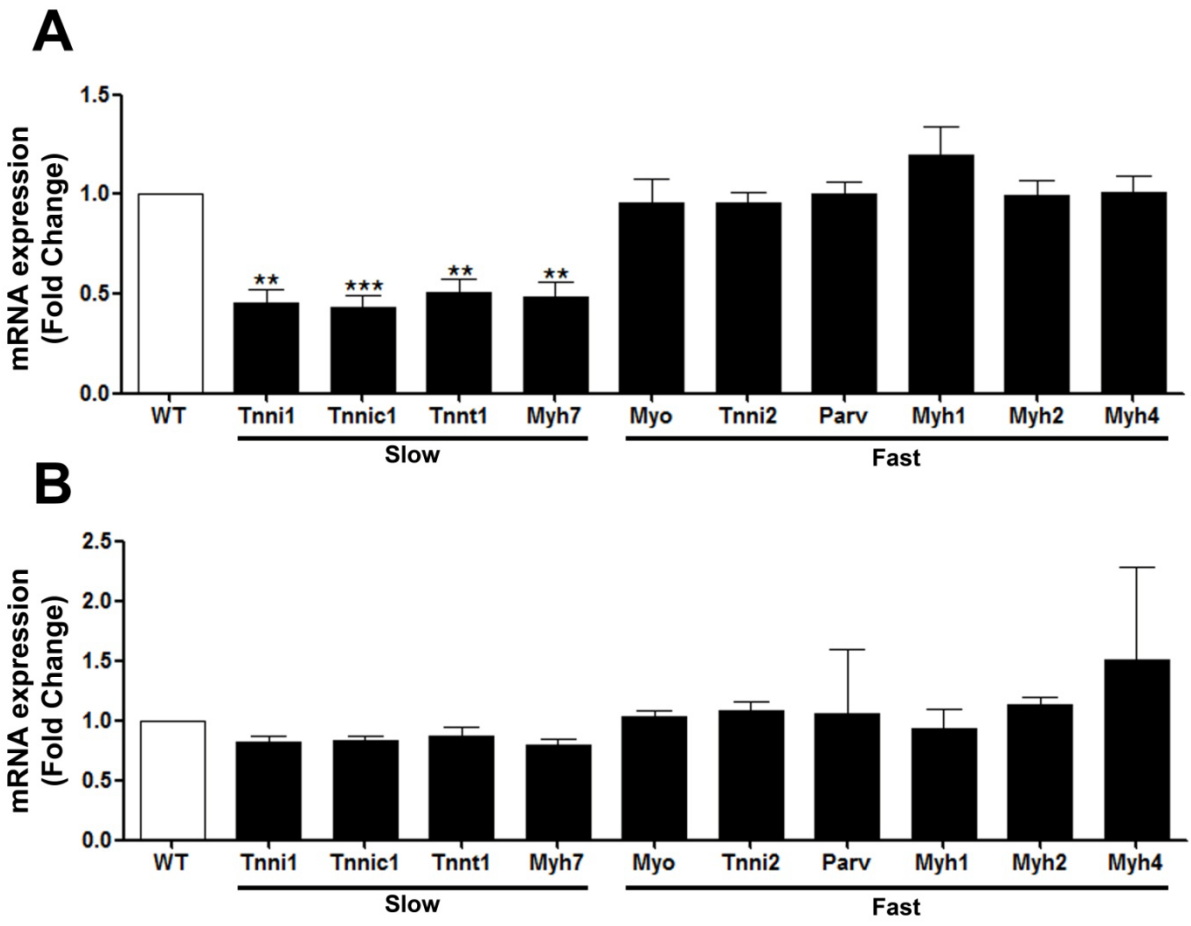

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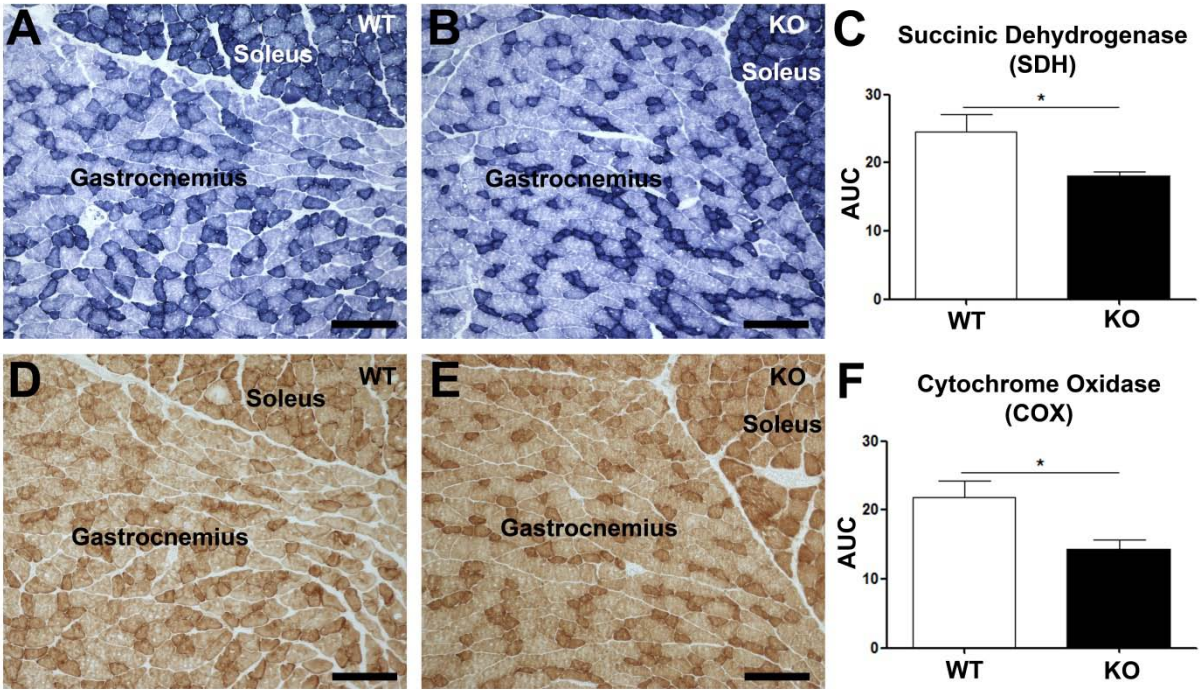

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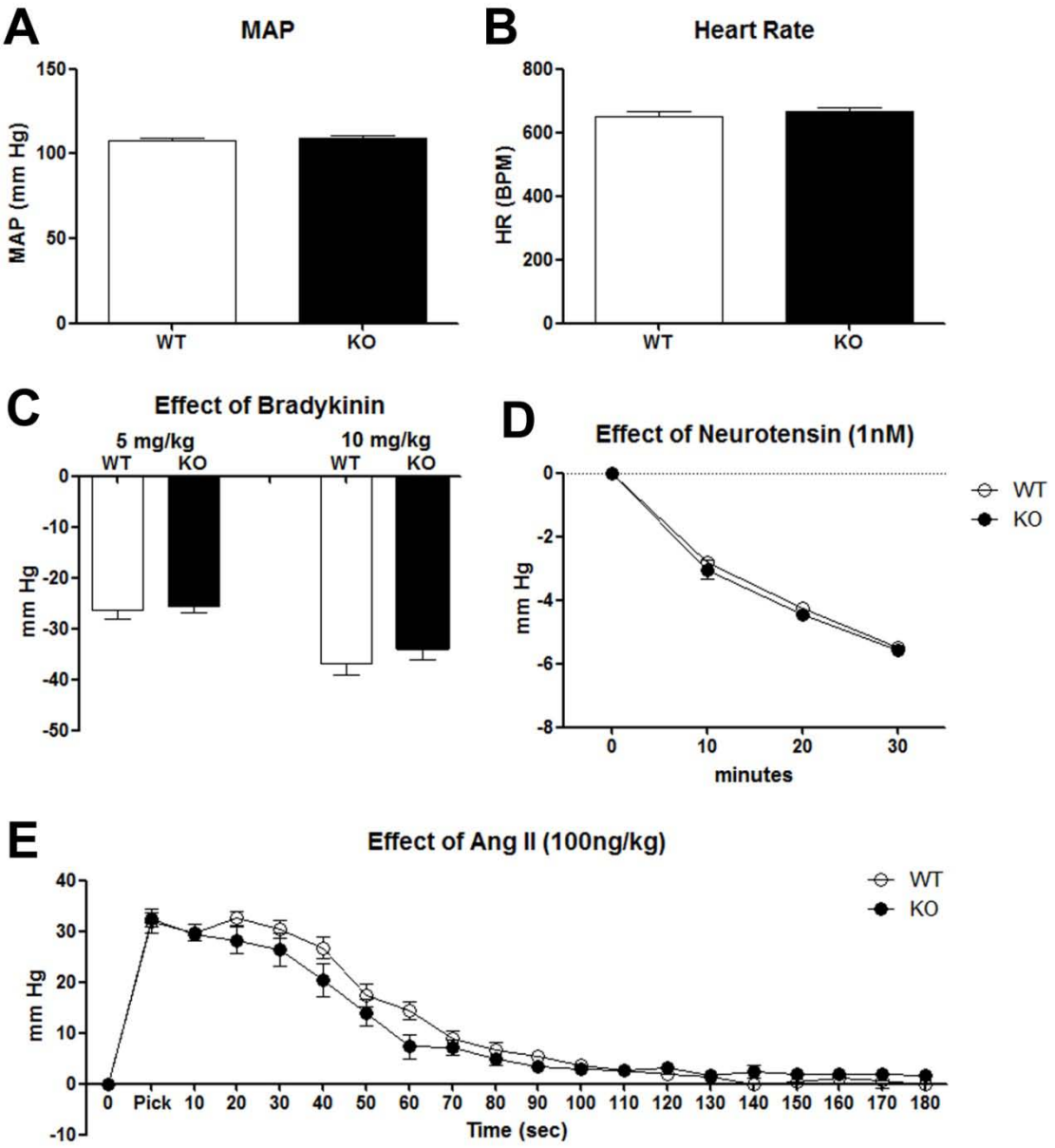

Figure 12