Neurolysin Knockout Mice Generation and Initial Phenotype Characterization

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Background: Neurolysin is known to cleave several bioactive peptides in vitro.

Results: Neurolysin knock-out 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.

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 KO mice have increased glucose tolerance, insulin sensitivity, and gluconeogenesis. KO mice have increased liver mRNA for several genes related to gluconeogenesis. Isotopic label semiquantitative peptidomic analysis suggests an 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.

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 acid residues that hydrolyzes only peptides containing 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 AT-T-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, because the enzyme has no signal peptide or a hydrophobic membrane-spanning domain.

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5 The abbreviations used are: Nln, neurolysin; ACE, angiotensin-converting enzyme; SDH, succinate dehydrogenase; COX, cytochrome oxidase; PGAM, phosphoglycerate mutase; CPSM, carbamoyl-phosphate synthase 1, mitochondrial; GGT, glucose tolerance test; ITT, insulin tolerance test; qPCR, quantitative PCR; GO, Gene Ontology; PPII, physical protein-protein interaction; ANOVA, analysis of variance; ESI, electrospray ionization; TMAB, 4-trimethylammoniumbutyryl.
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 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 knock-out mice (Cpefat/fat) show an obesity phenotype (33, 34), and dipeptidyl peptidase 4-deficient mice are resistant to developing 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 are 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 (29, 30). Nln activity is significantly lower in the fat tissue of transgenic mice that are 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 (29, 30).

EXPERIMENTAL PROCEDURES

Generation of Nln Knock-out Mice—The Nln gene trap knock-out mouse strain was generated by C57BL/6 blastocyst microinjection of genetically modified embryonic stem cells (NPX481, 129ola) obtained from Baygenomics through the International Gene Trap Consortium. The genetically modified Nln allele of the NPX481 strain has the gene trap vector pGT1dTMpfs inserted between exon 1 and exon 2 (Fig. 1A), as evidenced by sequencing 5’-H11032 rapid amplification of cDNA ends cDNA from the original NPX481 embryonic stem cell line (CC178547) used to generate this mice strain (a detailed report about this embryonic stem cell line can be found at the International Gene Trap Consortium website). After transmission of the knock-out allele from chimera to F1 generation, Nln mice were obtained from heterozygous breeding, and the line was further maintained on the mixed...
**Neurolysin KO Mice**

background by breeding Nln\(^{+/−}\) with Nln\(^{+/−}\) 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.

The 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 number 44, page 86).

**Genotyping**—Genotyping of animals was performed using PCR with primer NlnF3 (5′-CGCCTCTGCACTTACCA), pNlnwtR3 (5′-ATTTGGCATTTAAGAGATCG), and pNlnkoR2 (5′-CGTCTTCAACAACACACTCC). 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′-CTGAAGGCTGCAAGTAC-3′) and Nln-Rv (5′-CGCATGTTGTATTTCCT-3′).

**Enzymatic Activity**—The enzymatic activity of Nln was determined in triplicate using a continuous assay with a quenched fluorescent substrate (7-methoxycoumarin-4-acetyl-P-L-G-P) determined in triplicate using a continuous assay with a quenched fluorescent substrate (7-methoxycoumarin-4-acetyl-P-L-G-P-termined in triplicate using a continuous assay with a quenched fluorescent substrate (7-methoxycoumarin-4-acetyl-P-L-G-P-term) 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 per minute normalized by protein concentration.

**Body Composition**—Body composition (free water, fat, and lean mass) was estimated in 6-month-old mice by dual energy x-ray absorptiometry using a Hologic QDR 4500 scanner (Hologic, Waltham, MA) as described previously (38).

**GTT, ITT, and Gluconeogenesis**—Mice were fasted for 12 h, and blood samples were taken before and at 15, 30, 60, 90, and 120 min after the injection of 2 g/kg glucose (GTT) or 0.75 unit/kg insulin (ITT). Gluconeogenesis was assessed by injecting 2 g/kg sodium pyruvate after 16 h fasting. In all three assays, glycemias were 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 units/kg) as previously described (39). The samples were immediately stored in liquid nitrogen.

For Western blot analyses, tissue lysates were homogenized in radioimmune precipitation assay 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 h in blocking buffer (Odysey; LI-COR Biosciences), the membranes were incubated with anti-Nln (rabbit polyclonal antibody; Abcam), anti-phospho-AKT (Ser-473), or anti-AKT (rabbit polyclonal antibody; Cell Signaling) overnight at 4 °C and then incubated with IRDye secondary antibody (Odysey; LI-COR Biosciences). The results were visualized in an Odyssey infrared imaging system (Odysey; LI-COR Biosciences) and was quantified by densitometry with Odyssey infrared imaging software (Odysey; 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 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 qPCR to determine mRNA expression in liver, muscle, and adipose tissue. Animals fasted for 12 h 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; Invitrogen). The total RNA was cleaned in a RNeasy mini kit (Qiagen), and the RNA integrity was assessed by electrophoresis on 1% agarose gels.

cDNA was synthesized from 1 μg 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−ΔΔCt) 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 [supplemental Table S1](#).

**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. For high intensity running, intensity of exercise was increased by 3 m/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. For low intensity running, we used the average speed in the high intensity running and calculated 70% of this result (21 m/min) and 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: 0.25-inch outer diameter and 0.012-inch inner diameter; Braintree Scientific, Braintree, MA) 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
To test blood pressure response to different drugs (1 nM neurotensin, 5 and 10 mg/kg bradykinin, and 100 ng/kg angiotensin II), 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 epididymal adipose tissue were collected and subjected to 10 s of microwave (1500 watts; Electrolux) radiation 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 0.5 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 15,000 g for 40 min at 4 °C. The resulting supernatants were collected and filtered through a Millipore membrane (molecular weight cutoff = 10,000; Amicon Ultra™; Millipore). The flow-through was applied to C18-like Oasis columns (Waters) (Fig. 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 M2e 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 and 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 Me2SO 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

![Figure 2: Scheme for Intracellular Peptide Extraction, Labeling, and LC-MS/MS Analysis](image-url)
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 mM 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 mM 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). The peptides were applied to C18-like Oasis columns (Waters), eluted with 100% methanol, 0.15% trifluoroacetic acid, and dried in a vacuum centrifuge. Samples were resuspended in 10 μl of deionized water.

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 13C were used. Multiple scans of the MS spectra were combined prior to quantitation (Fig. 2).

Gene Ontology Analysis—The 40 proteins obtained from the peptidomic analyses shown in supplemental Table S3 plus Nln (shown in supplemental Table S4) were subjected to Gene Ontology (GO) clustering analysis using Biological Network Gene Ontology (BiNGO) software (50), a freely available plugin for the Cytoscape software, version 3.0.1 (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 false discovery rate control (85) 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 PPPI network containing Nln and the proteins represented by peptides from the peptidomic analyses shown in supplemental Table S3, we initiated data mining of human protein-protein interaction databases using them as a seed list. This procedure was carried out inside the Cytoscape platform with the APID2NET Cytoscape Plug-in software (54). The obtained network was named Union (because it was originated by the union of proteins identified in the gastrocnemius, liver, soleus, and adipose tissue samples), which was then submitted to GO analysis again, as above described.

Statistics—The values are expressed as means ± S.E. 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 Knock-out of Nln Expression in Mice—The summarized strategy of generating Nln KO mice by insertion of a gene trap between exons 1 and 2 is illustrated in Fig. 1A. The presence of the gene trap in the Nln gene of the knock-out mice was confirmed by genotyping (Fig. 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 (Fig. 1, C–E).

Initial Phenotypical Characterization of Nln Knock-out 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 WT C57BL/6 littermates 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 (Fig. 3, A–C). In contrast, KO mice showed a slight although statistically significant (p < 0.05) ~10% decrease in body weight compared with WT (Fig. 3D); however, no difference in the specific mass of the internal organs was observed (supplemental Table S2). There was also no obvious difference in the morphology of skeletal muscle, liver, and epididymal adipose tissue stained with hematoxylin and eosin1 (Fig. 3, E–J).

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

Comparative analyses of several regulatory genes involved in liver gluconeogenesis were performed in WT and KO using qPCR. These results suggest that KO have increased liver mRNA for the Fbp1 (fructose bisphosphatase 1), PPARγ (peroxisome proliferator-activated receptor γ), and Creb1 (cAMP-responsive element-binding protein 1), and Cox4i1, which is in agreement with an increased liver gluconeogenesis of KO when compared with the WT animals (Fig. 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 3 m/min until exhaustion. These results showed no statistical difference between WT and KO animals (Fig. 6, A and B); on average, WT and KO mice ran 422 and 337 m, respectively, before complete exhaustion. Second, animals were subjected to a low intensity running regime (21 m/min constant) until exhaustion, and the KO (637 m) ran significantly (~36%) less when compared with WT (997 m) (Fig. 6, C 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\(^{\text{Ser-473}}\) phosphorylation in the gastrocnemius, liver, and epididymal adipose tissues of both WT and KO animals. Whereas the basal levels of AKT\(^{\text{Ser-473}}\) phosphorylation were similar between WT and KO animals (Fig. 7, A–C), following a stimulus with an acute intravenous insulin injection, it was higher in gastrocnemius (Fig. 7A) and epididymal adipose tissue (Fig. 7B) but not in the liver (Fig. 7C) of KO compared with WT animals.

**FIGURE 3. Body weight, basal glucose levels, and body composition.** A–C, body composition of muscle mass (A), free water (B), and fat (C) (n = 6). D, average body weight of WT and 24-week-old KO animals (n = 6). The data are presented as means ± S.E. Student’s unpaired t test was used. *, p < 0.05. A–J, histological analysis. E and F, muscle histological sections (gastrocnemius) stained with hematoxylin and eosin of 24-week-old WT and KO animals, respectively. G and H, liver histological sections stained with hematoxylin and eosin of 24-week-old WT and KO animals, respectively. I and J, epididymal adipose tissue histological sections stained with hematoxylin and eosin of 24-week-old WT and KO animals, respectively (n = 5).
Fast Twitch Type II Fibers Remodeling in Nln Knock-out 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 (Fig. 8). Based on this finding and because 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 analyzed mRNA expression of specific genes for slow twitch type I (Tnni1, Tnnt1, Tnncl1, and Myh7) and fast twitch type II fibers (Tnni2, Pvalb, Myh1, Myh2, and Myh4) in gastrocnemius and soleus (Fig. 9, A and B). KO showed a decrease in mRNA expression typical for slow twitch type I fibers in gastrocnemius.

FIGURE 4. Glucose and insulin tolerance test. A, basal blood glucose levels after fasting for 12 h (n = 6). B, GTT after fasting for 12 h of 24-week-old WT and KO animals (n = 6). C, area under the curve (AUC) for the glucose tolerance test. D, after fasting for 12 h of 24-week-old WT and KO animals (n = 6). E, calculation of the constant of glucose disappearance (KITT) for the insulin tolerance test. The data are presented as means ± S.E. Student’s unpaired t test or ANOVA followed by Bonferroni’s test was used. *, 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-week-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). The data are presented as means ± S.E. Student’s unpaired t test or ANOVA followed by Bonferroni’s test was used. *, p < 0.05; **, p < 0.01; ***, p < 0.001. AUC, area under the curve.
but not in soleus, whereas no differences were observed for fast twitch type II fibers in both skeletal muscles (Fig. 9, A and B).

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

Distinctive Intracellular Peptide Profile of KO and WT in Skeletal Muscle, Liver, and Adipose Tissue—Semiquantitative 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 (supplemental Table S3). Altogether, 736 MS peptide spectra were quantified including those peptides that were not sequenced by MS/MS (supplemental Table S3). 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 2-fold (more than 100% of increase or more than 50% of decrease) in KO compared with WT mice; peptides that increase are considered potential Nln substrates, whereas peptides that decreased are considered potential Nln products. Thus, from the total of peptides identified (supplemental Table S3), 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 DMEVKVKQKSSKELEDMNQKL, increased more than 2-fold (supplemental Table S1B). In the adipose tissue two different peptides, one derived from the acyl-CoA-binding protein (VEKVDELKKKYGI) and one derived from the hemoglobin subunit α (LASVSTVLTSKYR), increased more than 2-fold (supplemental Table S3A). In the liver, several endocannabinoid peptides containing the mouse hemopressin (PVNFKLLSH) endocannabinoid signature (SDLHAHKLVDVPVNFK, RVDPVNFKLLS, VDPVNFKLL, VDPVNFKLLSH, and RVDPVN), or the previously identified antinociceptive AGH peptide (AGHLDLPGALSA) decreased more than 2-fold (supplemental Table S3C). In the soleus, only the peptide FASFPTTK derived from the hemoglobin α subunit decreased more than 2-fold (supplemental Table S3D). 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 ~5–20 amino acids. These data suggest a great specificity of Nln for metabolizing intracellular peptides and/or a compensatory mechanism occurring in the KO mice.

Nln Knock-out 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 versus 108.0 ± 1.5 mm Hg, respectively; Fig. 11A) and heart rate (668.4 ± 8.8 versus 652.0 ± 14.5 beats per min, respectively; Fig. 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 (Fig. 11, C–E). These data suggest that Nln alone is not a key enzyme in blood pressure regulation.

**Gene Ontology Analysis and PPPI Networks**—The intracellular peptides identified by LC-ESI-MS in the four peripheral tissues (adipose tissue, gastrocnemius, liver, and soleus) and shown in supplemental Table S3 were used to generate a list of thirty-nine proteins, which together with Nln itself were used for further in silico analysis (supplemental Table S4). To discover biological processes affected by the ablation of Nln, these proteins were submitted to 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, and CPSM) and muscle contraction (ALDOA, PGAM1, PGAM2, SMPX, and 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 (Fig. 12). These data suggest that Nln KO enhances CPSM and reduces PGAM1 and PGAM2 gene expression in the gastrocnemius, which is not observed in the soleus (Fig. 12).

Moreover, 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 40 proteins from supplemental Table S4 and constructed a network named Union (supplemental Fig. S1), because of the union of nonoverlapping proteins found in the gastrocnemius, liver, soleus, and adipose tissue samples, together with Nln. Despite the fact that 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 40 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 (supplemental Fig. S1). 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 knock-out of Nln (supplemental Table S5). In addition to the general and tissue-specific processes, Nln KO altered the expression of a number of genes involved in the regulation of glucose homeostasis, such as those encoding for enzymes involved in glycolysis and gluconeogenesis. These data suggest that Nln is not a key enzyme in blood pressure regulation, but it might play a role in the regulation of energetic metabolism.
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);

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). The data are presented as means ± S.E. Student’s unpaired t test was used. **, p < 0.01; *** p < 0.001.

FIGURE 10. Mitochondrial enzymatic activity in gastrocnemius. A and B, muscle histological sections showing the SDH staining of 24-week-old WT and KO animals, respectively. C, relative quantification of histochemical succinate dehydrogenase staining (n = 4). D and E, muscle histological sections showing COX staining of 24-week-old WT and KO mice, 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). The data are presented as means ± S.E. Student’s unpaired t test was used. *, p < 0.05. AUC, area under the curve.
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(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 KO mice. 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 suggest a novel therapeutic possibility, which

TABLE 1
Summary of intracellular peptides quantification by LC-ESI-MS
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). The percentages indicate the amounts of intracellular peptides that are substrates, products, or not cleaved by Nln (n = 5).

| Tissue          | 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%)                 |

FIGURE 11. Blood pressure. A and B, mean arterial pressure (A, MAP) (expressed in mm Hg) and heart rate (B, 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 (1 nm/mouse) (n = 8). E, time course of the effect of angiotensin II (Ang II) on MAP after angiotensin II injection in femoral vein (100 ng/kg) (n = 8). The data are presented as means ± S.E. Student’s unpaired t test or ANOVA followed by Bonferroni’s test shows no different statistical significance between groups.
induces the translocation of GLUT4 to the plasma membrane which can be mediated through AKT phosphorylation, which important effect of insulin is the increase of glucose uptake, blocking glycogenolysis and gluconeogenesis (59). The most adipose tissue and decreasing hepatic glucose production by food intake, increasing glucose uptake by skeletal muscle and (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 by 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, Tnnc1, 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. Taken 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 ACE transgenic mice are not affected by losartan, an antagonist of the angiotensin II receptor, suggesting that the renin-angiotensin-transgenic mice is likely not responsible for this phenotype (35). In contrast, Nln activity is significantly lower in the fat tissue of ACE transgenic mice 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.

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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 showed 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, GDVNTDRPLL; and DBI, TVGDVNT-RPGLLL) 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 intracellular peptide profile and suggested that intracellular peptides could be endogenously involved in insulin-stimulated glucose uptake.

Previous biochemical, structural, and pharmacological evidence supports the high specificity of Nln for degrading small bioactive peptide substrates (9–11). Here, semiquantitative mass spectrometry analysis shows that specific intracellular peptides were affected in the KO compared with 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 β-adrenergic signal transduction in parallel to the intracellular peptide profile (73). Therefore, it is an exciting possibility to suggest that the phenotypes observed here in the KO mice are mediated by intracellular peptides caused by an oligopeptidase (77) action.

Nln have been suggested to participate in several important physiological processes like cardiovascular and renal homoestasis, pain perception, and 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 (24, 28, 78). Even though Nln is expressed widely in the central nervous system and 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 with 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 A1–8), 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 knock-out only affects the levels of Met-enkephalin and octapeptide and thus exerts only a minor effect on intracellular peptide metabolism in the brain (82). Altogether, these data suggest 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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