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Transthyretin knockout mouse nerves have increased lipoprotein lipase and sphingolipid content following crush

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ABSTRACT

Transthyretin (TTR) knockout (KO) mice display increased levels of lipoprotein lipase (LPL) and impaired nerve regeneration. Given LPL potential role in the reutilization of myelin lipids following injury, we compared myelin lipid content in wild-type and TTR KO mice after nerve crush. We found that LPL is expressed not only in Schwann cells but also in dorsal root ganglia neurons and that its activity is increased in TTR KO mice following nerve injury. As a possible consequence of LPL increase in the regenerating nerve of TTR KOs, the sphingolipids sphingomyelin and galactocerebroside were augmented in the distal nerve stump. Given their ability to increase neurite outgrowth, upregulation of LPL and sphingolipids in a system with decreased capacity for nerve regeneration probably constitutes a compensatory mechanism.

Transthyretin (TTR) is the plasma transporter of thyroxine and retinol [26]. It has been demonstrated from studies on TTR knockout (KO) mice [7] that TTR is additionally associated to nervous system functions, namely behaviour [28], neuropeptide processing [21] and nerve regeneration [9]. In relation to neuropeptide processing, TTR KO mice display increased expression of peptidylglycine α-amidating monooxygenase (PAM), the rate-limiting enzyme in amidated neuropeptide maturation, both in the peripheral nervous system (PNS) and central nervous system (CNS) [21]. Consequently, TTR KOs have increased amidated neuropeptide Y (NPY) and an NPY-overexpressor phenotype. This phenotype includes increased expression and activity of lipoprotein lipase (LPL), an enzyme induced by NPY [21]. In the PNS, it was additionally shown that TTR enhances axonal regeneration both *in vivo* and *in vitro*. Following nerve crush, TTR KO mice have a decreased number of axons and *in vitro*, neurite outgrowth is decreased in the absence of TTR [9]. Despite the carrier functions of TTR, TTR KOs are euthyroid and lack symptoms of vitamin A deficiency [7]. As such, the above effects have been linked to TTR and not to its ligands.

As mentioned, TTR KOs have increased LPL. This rate-limiting enzyme in triglyceride hydrolysis is synthesized in several tissues, including the nervous system, where its function remains unclear. The wide expression of LPL in the brain and its presence in spinal cord oligodendrocytes implicated it in myelination and maintenance of CNS myelin [2,22]. Sciatic nerves immunostained for LPL demonstrated a general endoneurial fluorescence, with periaxonal and perivascular staining, consistent with LPL synthesis by Schwann cells, macrophages and/or fibroblasts [13]. LPL expression by Schwann cells was further confirmed in cell culture experiments [12]. Furthermore, when given an exogenous triglyceride source, both cultured brain cells and Schwann cells incorporate the triglyceride-derived fatty acids into newly synthesized lipids [6,12]. This suggested that LPL provides free fatty acids for lipid biosynthesis thus probably playing a critical role in myelin maintenance. After sciatic nerve crush, LPL is increased in the distal nerve stump [13], suggesting that it participates in recycling myelin lipids, perhaps by facilitating free fatty acid uptake by Schwann cells to remyelinate the regenerating nerve.

Given the role of LPL in myelin lipids reutilization following nerve injury, and the fact that TTR KOs have increased LPL and impaired nerve regeneration, we compared myelin lipid content in WT and TTR KO mice after nerve crush.

Mice were handled according to European Union rules (86/609/EEC) and studies were approved by the Portuguese Veterinarian Board given that all protocols were developed in order to minimize animal pain and discomfort. Six-month old WT and TTR

Abbreviations: DRG, dorsal root ganglia; HPTLC, high-performance thin-layer chromatography; KO, knockout; LPL, lipoprotein lipase; NPY, neuropeptide Y; PAM, peptidylglycine α-amidating monooxygenase; TTR, transthyretin; WT, wild type.

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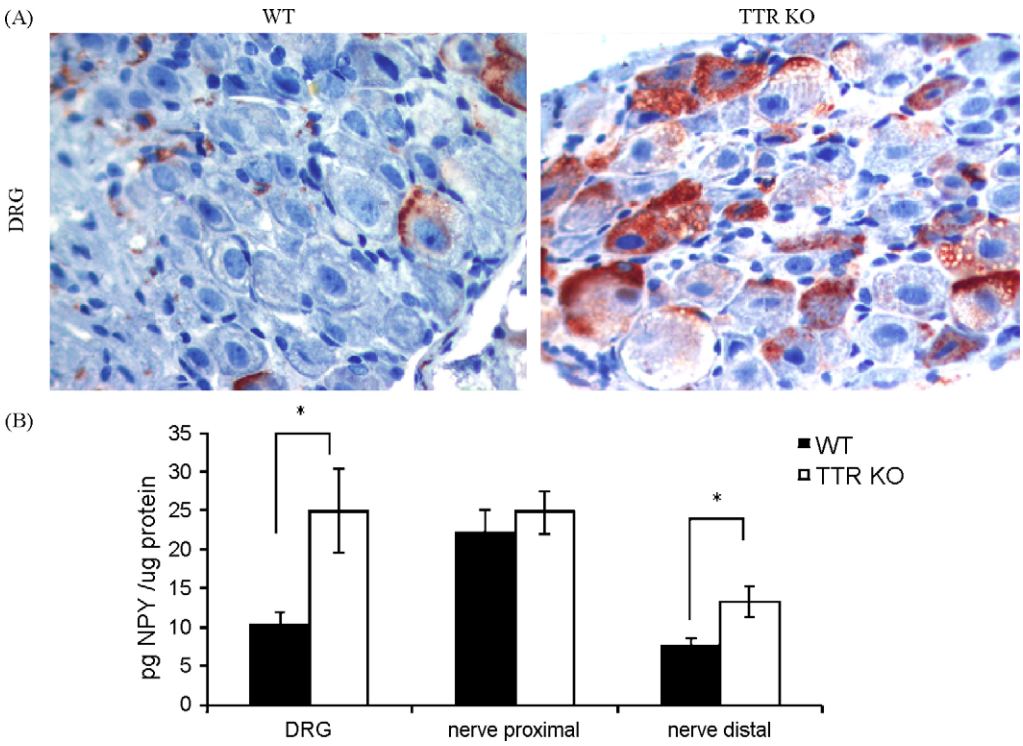

Fig. 1. PAM and NPY levels in WT and TTR KO mice 15 days following sciatic nerve crush. (A) PAM immunohistochemistry in DRG (40× magnification). (B) NPY content in the DRG and proximal and distal sciatic nerve stumps. Results are presented as average ± S.E.M.; $^{*}P < 0.05$.

KO [7] littermates were used in groups of 6 animals/genotype, sex matched.

Sciatic nerve crush was performed bilaterally in a constant site using Pean forceps, twice during 15 s, as described [9]. Animals recovered for 15 days, after which the dorsal root ganglia (DRG) L5 and proximal and distal sciatic nerve stumps were removed.

Incubation with primary antibodies diluted in blocking buffer (1% BSA and 4% horse serum in PBS) was performed overnight at 4 °C. Primary antibodies were rabbit anti-PAM (Ab246, from Dr. Mains, University of Connecticut Health Center, 1:500) and a rabbit anti-LPL that, according to the manufacturer's instructions, shows no cross-reactivity with other LPL family members (Cell Applications, CA, USA, 1:50). Antigen visualization was performed with the biotin–extravidin-peroxidase kit (Sigma, Portugal). On control sections, the primary antibody was omitted. Analysis was performed independently by two observers and labeling scored from 0 (none) to 3 (intense).

Quantification of amidated NPY was performed by radioimmunoassay using the Bachem (Germany) kit [21]. For peptide extraction from DRGs and sciatic nerves, samples were boiled in 10 V of extraction buffer (2 M acetic acid and pH 3.4), homogenized and sonicated. Supernatants were lyophilized and pellets were used for total protein quantification with BioRad (Bio-Rad, Portugal). Lyophilized samples were dissolved in RIA buffer and the assay was performed following the instructions. NPY content was normalized per total protein.

LPL activity assays were performed using the Total Lipase Test kit (Progen Biotechnic, Germany) [21]. Although this measures total lipase, hepatic lipase and pancreatic lipase are absent in the nerve where only LPL is expressed. Nerve stumps were homogenized in Krebs–Ringer's buffer, and heparin (Sigma) was added to a final concentration of 100 U/mL. After 45 min incubation at 37 °C and centrifugation at 14,000 × *g*, supernatants were used in the assay. LPL activity was normalized per tissue weight.

Sciatic nerve crush was performed as described above. Following 15 days of regeneration, distal nerve stumps were collected and processed for quantification of free fatty acids using the enzyme-based free fatty acid quantification kit (BioVision, CA, USA), according to the manufacturer's instructions. Briefly, tissue samples were extracted with 200 μL chloroform–1% Triton X-100 in a microhomogenizer and the organic phase was used for free fatty acid quantification. Free fatty acid content was normalized per total protein (quantified using the BioRad reagent).

Analysis of sciatic nerve myelin lipids was performed by isolating total lipids [10]. This procedure yields an accurate estimate of myelin lipids, as these are much more abundant than lipids from other sources of membrane in the nerve [10]. After nerves were homogenized, lipids were extracted with chloroform:methanol (1:2), fractionated in C18 columns (Macherey-Nagel, Germany) [17] and separated in HPTLC silica gel plates (Merck, Portugal) [29]. For each analysis, the same tissue weight was spotted per lane. Phosphatidylethanolamine and phosphatidylcholine were resolved with chloroform:methanol:acetic acid:formic acid:water (35:15:6:2:1). To separate cholesteryl esters from triglycerides a second dimension with *n*-hexane:diisopropyl ether:acetic acid (65:35:2) was performed [19]. Galactocerebroside and sphingomyelin were separated by spraying the plates with 1% sodium perborate before sample application and resolution with chloroform:methanol:water (70:30:5) [15]. Gangliosides were resolved with chloroform:methanol:15 mM calcium chloride (55:45:10). All solvents were analysis grade (Merck). A known amount of the corresponding lipid standards (Sigma) was applied. Lipid spots were visualized with 1% anisaldehyde in sulfuric acid 80%, except for gangliosides in which 0.3% resorcinol, 80% hydrochloric acid and 0.25 mM copper sulfate were used. Quantification was performed with UN-SCAN-IT software (Silk Scientific, USA) and results were normalized per tissue weight.