KELLY VALÉRIO PRATES

EFEITOS DA ABLAÇÃO DO SISTEMA NERVOSO SIMPÁTICO NA SECREÇÃO DE INSULINA E NA PROGRAMAÇÃO METABÓLICA

Maringá
Janeiro - 2017

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Dissertação apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Mestre em Ciências Biológicas.

Prof(ª). Dr(ª). Paulo Cezar de Freitas Mathais
Orientador

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Dissertação apresentada à Universidade Estadual de Maringá, como requisito parcial para a obtenção do título de Mestre em Ciências Biológicas.

Aprovada em 03/03/2017

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Kelly Valério Prates nasceu em Maringá/PR em 10/05/1986. Possui Graduação em Ciências Biológicas pela Universidade Estadual de Maringá (2014). Tem experiência na área de Biologia Celular e Bioquímica, atuando principalmente nos seguintes temas: Conceito DOHaD, Programação Metabólica, Secreção de Insulina, Sistema Nervoso Autonômico.

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DEDICATÓRIAS

À minha mãe, *Francisca Valério*, mulher batalhadora, um exemplo para mim.

APRESENTAÇÃO

Essa dissertação é composta de um artigo científico, apresentando os temas sistema nervoso simpático e secreção de insulina. Por meio de estudo funcional, demonstramos que a ablação de nervos autonômicos que inervam o pâncreas e que estão intimamente relacionados ao controle da secreção de insulina, conduzem a alterações fisiológicas e fisiopatológicas. Interessantemente, observamos que, a intervenção cirúrgica, através da simpatectomia pancreática, ainda que realizado na vida adulta, é capaz de desencadear alterações metabólicas, principalmente relacionados a alterações nos mecanismos que controlam a secreção de insulina, propiciando o aparecimento de doenças como o diabets tipo 2. Em consonância com as regras do Programa de Pós-Graduação em Ciências Biológicas, o artigo entitulado ‘‘*Sympathetic innervation is essential for metabolic homeostasis and pancreatic beta cell function in adult rats’’* foi publicado na revista *‘‘Molecular and Celular Endocrinology* (print)*’’*, com Qualis A2 na área de Ciências Biológicas I e Fator de Impacto 3,754.

Manuscrito

Kelly V. Prates; Júlio C. de Oliveira; Ananda Malta; Camila C. I. Matiusso; Rosiane A. Miranda; Tatiane A. Ribeiro; Flávio A. Francisco; Claudinéia C. S. Franco; Veridiana M. Moreira; Vander S. Alves; Rosana Torrezan; Paulo C. F. Mathias; Luiz F. Barella.

‘‘**Sympathetic innervation is essential for metabolic homeostasis and pancreatic beta cell function in adult rats’’.**

RESUMO GERAL

INTRODUÇÃO - O sistema nervoso autonômico (SNA) desempenha um papel crucial na regulação da homeostase metabólica. Esta regulação inclui o controle da secreção de insulina pelo pâncreas endócrino, através da estimulação pelos receptores colinérgicos muscarínicos, e/ou inibição da secreção via receptores adrenérgicos. O desequilíbrio na atividade do SNA, com o aumento da atividade do sistema nervoso parassimpático (SNP) e/ou diminuição da atividade do sistema nervoso simpático (SNS), tem sido sugerido como um fator de risco para o desenvolvimento e manutenção de disfunções metabólicas, como o diabetes tipo 2.

OBJETIVOS - Objetivamos investigar se o desequilíbrio do SNA induzido por simpatectomia pancreática pode conduzir a distrúrbios metabólicas, incluindo modificações na secreção de insulina pelas células beta-pancreáticas.

MÉTODOS – Para a realização desse estudo utilizamos ratos da linhagem Wistar, os quais foram submetidos a simpatectomia pancreática aos 60 dias de vida, através da excisão unilateral do nervo esplâncnico que se projeta para o pâncreas. A evolução do peso corporal foi mensurada o período da eutanásia, aos 120 dias de vida. Glicemia e insulinemia foram mensurados pelo teste de tolerância à glicose intravenoso (TTGiv). Os estoques de gordura (retroperitoneal, mesentérica e periepididimal) foram removidos e pesados para avaliar o estado de obesidade. Para melhor compreensão de possíveis alterações metabólicas ocorridas no pâncreas endócrino, as ilhotas pancreáticas foram incubadas com glicose na presença ou na ausência de acetilcolina (ACh); bem como, com drogas que bloqueiam os receptores colinérgicos muscarínicos [antagonistas seletivos para o subtipo M_2mAChR (Metoctramina, MTT) ou M_3mAChR (4-Diphenylacetoxy-N-methylpiperidine methiodide, 4-DAMP). Outro grupo de ilhotas, foram incubadas para estudar a contribuição dos receptores adrenérgicos no controle da secreção de insulina. Para tanto, utilizamos epinefrina (Epi); bem como, yohimbine (Yoh) ou propranolol (Pro) [antagonistas dos receptores alfa-2 adrenérgico (α2-AR) e beta-adrenérgico (β-AR), respectivamente]. A expressão proteica do receptor α2-AR foi realizada pela técnica de *western blotting*. Para as análises estatísticas utilizamos o programa GraphPad Prism versão 6.01 para Windows (GraphPad Software Inc., San Diego, CA, USA).

Os resultados são expressos como média ± EPM e submetidos ao teste *t de Student*, ANOVA de medidas repetidas ou ANOVA de uma via. Os valores de $p < 0.05$ foram considerados estatisticamente significantes.

RESULTADOS E DISCUSSÃO – Os ratos simpatectomizados (SYM) apresentaram uma modesta elevação de peso corporal, bem como, aumento do acúmulo dos principais estoques de gorduras aos 120 dias de vida. Esses resultados em conjunto, indicam que a simpatectomia pancreática realizada no início da vida adulta prejudicou o balanço energético do metabolismo. Apesar de não ter ocorrido alterações na glicemia de jejum, observamos diminuição da secreção de insulina no jejum, bem como, um aumento da sensibilidade periférica à insulina no grupo SYM. Nosso modelo experimental demonstrou prejuízo na secreção estática de insulina em presença de glicose. Nossos resultados revelam uma maior resposta da secreção de insulina em presença de ACh e de MTT (23% e 105%, respectivamente), sem nenhuma modificação em presença de 4-DAMP. A respeito dos dados supracitados, hipotetizamos que possa ter havido um desbalanço na função e/ou composição dos receptores muscarínicos dos subtipos M2mAChR e M3mAChR nas ilhotas pancreáticas do grupo SYM. Verificamos uma fraca resposta da secreção de insulina, quando tratamos as ilhotas com Epi, com diminuição de 24%; embora não observamos nenhuma modificação da secreção de insulina com os antagonistas adrenérgicos, Yoh e Pro. Constatammos elevação de 43% na expressão proteica dos α2-AR do grupo SYM; esse resultado, nos revela que a inervação intacta do SNS do pâncreas endócrino exerce forte influência sobre a função e/ou expressão dos receptores adrenérgicos. Modelos experimentais em roedores transgênicos, demonstram que a ausência de receptores β-AR poderia desencadear o aumento na expressão dos α2-AR, o que causaria impactos na secreção de insulina. Em contrapartida, modelo transgênico de roedores que superexpressam os α2-AR, foram observados perda da homeostase glicêmica e diminuição na secreção de insulina. Apesar da elevação dos α2-AR na célula-beta, não observamos aumento da secreção de insulina no estudo *in vitro* com Yoh. Contudo, é presumível hipotetizar que possa ter ocorrido alteração na função e/ou expressão de outros receptores adrenérgicos, como um possível mecanismo de contrabalancear a superexpressão dos α2-AR. Estudos futuros são necessários para melhores compreensões do mecanismo que envolve o controle da secreção de insulina através dos receptores adrenérgicos.

CONCLUSÃO – A ablação do SNS no início da vida adulta conduziu ao desbalanço

na atividade do SNA, comprometendo o metabolismo energético. Acompanhado por perda da homeostase glicêmica e danos no controle da secreção de insulina tanto na presença de glicose, como em presença de agentes muscarínicos e adrenérgicos, e ainda, superexpressão dos α2-AR. Desequilíbrio do SNA, ainda que na vida adulta, desencadeia disfunções metabólicas, principalmente atráves de alterações nos mecanismos que regulam o fino controle da secreção de insulina. Ademais, diminuição da secreção de insulina por superexpressão dos α2-AR podem participar do desenvolvimento do diabetes tipo 2.

GENERAL ABSTRACT

INTRODUCTION – The autonomic nervous system (ANS) plays a crucial role in the regulation of metabolic homeostasis. This regulation includes the control of insulin release by the endocrine pancreas, through stimulation by muscarinic cholinergic receptors, and/or inhibition of insulin release by adrenergic receptors. The imbalance in ANS activity, through increased activity of the parasympathetic nervous system (PNS) and/or reduction of sympathetic nervous system (SNS) tone, has been suggested as a risk factor for the development and maintenance of metabolic dysfunction, such as type 2 diabetes.

AIMS – We aimed investigate whether the ANS imbalance induced by pancreatic sympathectomy can lead to metabolic alterations, including changes in insulin release from pancreatic beta-cells.

METHODS – For this study we used Wistar rats, which were submitted to selective pancreatic sympathectomy at 60-day-old, through unilateral excision of the splanchnic nerve that projects to the pancreas. The evolution of body weight and food intake were collected until the euthanasia period, at 120-day-old. Glycemia and insulinemia were measured by the intravenous glucose tolerance test (ivGTT). Fat pad stores (retroperitoneal, mesenteric and periepididymal) were removed and weighed to assess the state of obesity. Towards a better understanding of the changes in the endocrine pancreas, pancreatic islets were incubated with glucose in the presence or absence of acetylcholine (ACh), as well, as with drugs that block muscarinic acetylcholine receptors [selective antagonist to M2mAChR subtype (Metoctramine, MTT) or M3mAChR subtype (4-Diphenylacetoxy-N-methylpiperidine methiodide, 4-DAMP)]. Another group of islets were incubeted to study the contribution of adrenergic receptors in the control of insulin secretion. For this, we used epinephrine (Epi); as well, yohimbine (Yoh) or propranolol (Pro) (adrenergic receptor alpha-2 (α2-AR) - and beta antagonists (β-AR), respectively). Protein expression of alpha-2 adrenergic receptor was performed by the western blotting. For statistical analysis we used the GraphPad Prism version 6.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). The results are given as the means ± SEM and submitted to Student's t-test, ANOVA repeated measures or ANOVA One-way. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION – Sympathectomized rats (SYM) presented increased food intake, followed by a modest elevation in body weight, and an increase in the accumulation of the main fat stores at 120-day-old. These results together indicate that pancreatic sympathectomy performed early in adult life impaired energy metabolism. Although fasting glycemia was unchanged, we observed a 10% increase in glycemia in the area under the curve (AUC) of ivGTT; moreover, we observed fasting hypoinsulinemia and 30% decrease in insulin AUC during the test. We found high insulin sensitivity in a fasting condition, calculated through HOMA-IR index, the ablation of sympathetic innervation that protrudes into the pancreas, was able to cause loss in glycemic control, causing glucose intolerance, probably due to defect in glucose-stimulated insulin secretion. Similar to the *in vivo* data, we observed that the static secretion of insulin in the presence of glucose was also impaired. Our results revealed a higher insulin secretion response in the presence of ACh and MTT (23% and 105%, respectively), and no change in presence of 4-DAMP. About the above-mentioned data, we hypothesized that there may have been an imbalance in the function and/or composition of the muscarinic receptors of the M2mAChR and M3mAChR subtypes in the pancreatic islets of the SYM group. We observed a poor response of insulin secretion when we treated the islets with Epi, the decrease was 24%. Although we did not observe any modification of insulin secretion with the adrenergic antagonists, Yoh and Pro. We found a 43% increase in the protein expression of the alpha-2 adrenergic receptors of the SYM group, this result reveals that the intact innervation of the SNS of the endocrine pancreas exerts a strong influence on function and/or expression of adrenergic receptors. Experimental models in transgenic rodents, demonstrate that the absence of beta-adrenergic receptors could trigger increased expression of α2-AR, which would impact on insulin secretion. In contrast, another transgenic model that overexpressed α2-AR, loss of glycemic control and decrease in insulin secretion were observed. Despite significant elevation of α2-AR in the beta-cell, we did not observe an increase in insulin secretion in the isolated pancreatic islet incubation with Yoh. However, it is presumed hypothesized that there may have been alterations in the function and/or expression of other adrenergic receptors, such as a possible mechanism to counterbalance the overexpression of α2-AR. Future studies will be needed to better understand this interesting mechanism that involves the control of insulin secretion through adrenergic receptors.

CONCLUSIONS – SNS ablation in early adulthood led to the imbalance in the activity of the ANS, compromising the energy metabolism. Accompanied by loss of glycemic homeostasis and damage to the control of insulin secretion to either glucose or muscarinic/adrenergic agents, and overexpression of α2-AR. SNA imbalance, although in adult life, triggers metabolic dysfunctions, mainly through alterations in the mechanisms that regulate the fine control of insulin release. In addition, decreased of insulin release by overexpression of α2-AR may participate in the development of type 2 diabetes.

TEXTO REFERENTE AO ARTIGO

(Text for the article)

SYMPATHETIC INNERVATION IS ESSENTIAL FOR METABOLIC HOMEOSTASIS AND PANCREATIC BETA CELL FUNCTION IN ADULT RATS

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Short title: Sympathectomy and metabolic homeostasis
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* These authors equally contributed to this work.

Abstract

Obesity is associated with an imbalance in the activity of the autonomic nervous system (ANS), specifically in the organs involved in energy metabolism. The pancreatic islets are richly innervated by the ANS, which tunes the insulin release due to changes in energy demand. Therefore, changes in the sympathetic input that reach the pancreas can lead to metabolic dysfunctions. To evaluate the role of the sympathetic ends that innervate the pancreas, 60-day-old male Wistar rats were subjected to sympathectomy (SYM) or were sham-operated (SO). At 120 day-old SYM rats exhibited an increase in body weight, fat pads and metabolic dysfunctions. Decreases in the HOMA-IR and reductions in insulin release were observed both *in vivo* and *in vitro*. Furthermore, the SYM rats exhibited altered pancreatic islet function in both muscarinic and adrenergic assays and exhibited high protein expression of the alpha-2 adrenergic receptor (α2AR). Because α2AR has been linked to type 2 diabetes, these findings demonstrate the clinical implications of this study.

Keywords: autonomic nervous system, sympathectomy, endocrine pancreas, insulin release, metabolism, alpha-2 adrenergic receptor

Abbreviations: 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; ACh, acetylcholine; α2AR, alpha-2 adrenergic receptor; ADRA2A, alpha-2 adrenergic receptor gene; ANS, autonomic nervous system; AUC, area under the curve; βAR, beta-adrenergic receptor; BW, body weight; HBSS, Hank's buffered saline solution; HOMA-IR, homeostasis model assessment of insulin resistance; ivGTT, intravenous glucose tolerance test; mAChR, muscarinic acetylcholine receptor; MTT, methoctramine; Neo, neostigmine; PNS, parasympathetic nervous system; Pro, propranolol; RIA, radioimmunoassay; SO, sham-operated; SYM, sympathectomized; SNS, sympathetic nervous system; Yoh, yohimbine.

1. Introduction

The global population is currently witnessing a burden of obesity and associated metabolic disorders, such as type 2 diabetes (Chen et al., 2012; Stein & Colditz, 2004). Although obesity is considered to be caused by several factors (Haslam & James, 2005; Martinez, 2000), several years previously, it was proposed that an imbalance of the autonomic nervous system (ANS) could play a key role in its development and maintenance (Bray, 1991). In general, this imbalance is observed by high parasympathetic activity accompanied by low sympathetic tonus, specifically in organs involved in energy metabolism (de Oliveira et al., 2011). Several obese animal models and obese humans presented this imbalance as a potential disruption in metabolic homeostasis, such as by stimulating the release of high levels of insulin by pancreatic beta cells (Bray & York, 1979; Davy & Orr, 2009). The endocrine pancreas is innervated by the ANS, and the parasympathetic and sympathetic branches are responsible for stimulating and inhibiting insulin release in response to changes in energy demand. In pancreatic islets, the parasympathetic nervous system (PNS) releases acetylcholine (ACh), and ACh then acts on the four subtypes of muscarinic receptors expressed in the pancreas (M1mAChR-M4mAChR), which belong to the family of G-protein coupled receptors (Miranda et al., 2014). In contrast, the sympathetic ends release noradrenaline, which acts through the activation of the alpha-2 adrenergic receptor (α2AR) (α-2A, α-2B and α-2C isoforms) and beta-adrenergic receptor (βAR) (β-1, β-2, and β-3 isoforms) that are also G-protein coupled receptors. (Ahrén et al., 2006; Burris & Hebrok, 2007; Fagerholm et al, 2011). Regarding adrenergic signalling in beta cells, while the binding of noradrenaline to α2AR inhibits insulin release, the activation of the βAR by catecholamines enhances insulin release; nevertheless,

α2AR is predominantly expressed in the pancreatic islets (Ahrén, 2009; Fagerholm et al., 2011; Winzell & Ahrén, 2007). There are unresolved questions about the mechanisms of control and the interaction between both branches of the ANS in the pancreatic islets. At 60 days of age, Wistar rats are considered adults and have thus completed the development of the fibres and connections of the ANS in the endocrine pancreas (Borden et al., 2013). We hypothesize that sympathetic denervation of pancreatic islets in adult rats leads to changes in the ANS interaction and/or the fine-tuning control of the adrenergic receptor on insulin release. To assess the role of sympathetic innervation in the pancreas on body weight gain, glucose-insulin homeostasis and autonomic regulation upon insulin release, as well as α2AR expression in pancreatic islets, we denervated the splenic-sympathetic branches in 60-day-old rats. Our findings suggest that intact sympathetic nervous system (SNS) fibres may be a predominant factor involved in insulin release and, consequently, energy metabolism in adult life.

2. Material and Methods

2.1 Animals and ethical approval

All experiments were approved by the Ethics Committee in Animal Research of the State University of Maringa (protocol number 104/2012). At 60 days of age, male Wistar rats were randomly chosen for the study and distributed into two experimental groups (n=15; minimum 3 litters for each experimental group), which were supplied by the central animal facility of the State University of Maringa. Rats were maintained in cages (5 rats per cage) and kept under controlled conditions (temperature: 22 ± 2°C; photoperiod: 07:00 a.m. to 07:00 p.m.), with water and

standard chow diet (*Nuvital*®, Curitiba/PR, Brazil) provided *ad libitum* throughout the experimental period.

2.2 Alteration in the adrenergic system by sympathectomy surgery

The 60-day-old Wistar rats, in the fed state, were randomly chosen and distributed into two experimental groups (n=15; minimum 3 litters for each experimental group). The rats were anaesthetized by an intramuscular injection of xylazine and ketamine (0.6 mg and 3 mg/100 g body weight (BW), respectively). Next, the rats underwent an incision of the abdominal wall, and the stomach and intestine were retracted to expose the region between the pancreas and kidney that is the exact *anatomical* location of the splenic sympathetic nerve (Larson et al., 1985; de Souza, 2003; Sun et al., 2015; Xiong et al., 2011). In the SYM rat group, the branches of the left splenic sympathetic nerve were gently isolated and cut, but in the sham-operated group (SO), the branches were left intact.

Following surgery, the muscular and epidermal layers of the animals were sutured, and skin antisepsis was performed with 10% povidone-iodine. All rats received post-surgical treatment with analgesic as previously described (Mickley et al., 2006).

2.3 Glucose and insulin metabolism

At 120 days old, rats of both experimental groups (n=15, minimum 3 litters for each experimental group), were anaesthetized via an intramuscular injection of xylazine and ketamine (0.6 mg and 3 mg/100 g BW, respectively), and a silicone cannula (pre-treated with 50 IU heparin/ml of 0.9% saline solution to prevent blood clots) was implanted in the right jugular vein and fixed at the back of the neck. One

day after the surgery, the rats underwent a 12-h fast prior to receiving an infusion of glucose (1 g/kg BW) through the cannula, without anaesthesia. The blood samples were collected immediately before the glucose load (time zero/basal) and at 5, 15, 30 and 45 min after the glucose load (Ribeiro et al, 2016). The plasma obtained from the blood samples was stored at -20°C until the glucose concentration was measured by the glucose oxidase method using commercial kits (Gold Analisa®, Belo Horizonte, MG, Brazil). The insulin concentrations were determined by an ^{125}I-labeled insulin radioimmunoassay (RIA) using a gamma counter (PerkinElmer®, Shelton, CT, USA), with human insulin as the standard and an antibody against rat insulin as the tracer (Scott et al., 1981). Insulin intra- and inter-assay variation coefficients were 9.8% and 12.2%, respectively. The inferior and superior detection limits were 0.006 ng/ml and 100 ng/ml, respectively. Total glycaemia and insulinemia increases were calculated using the glycaemia and insulinemia AUC for the 45 min of the ivGTT.

2.4 Homeostasis model assessment of insulin resistance (HOMA-IR)

The HOMA-IR was calculated with the fasting glycaemia and insulinemia data using a HOMA-IR calculator provided by the Oxford Centre for Diabetes, Endocrinology and Metabolism (Wallace et al., 2004).

2.5 Isolation of pancreatic islets

Pancreatic islets from 120-day-old rats were isolated via the collagenase method as previously described (de Oliveira et al., 2013). Hank's buffered saline solution [HBSS, (mmol/l): NaCl, 136.9; KCl, 5.4; $MgSO_4 7H_2O$, 0.81; Na_2HPO_4, 0.34; KH_2PO_4, 0.44; $CaCl_2 2H_2O$, 1.26; $NaHCO_3$, 4.16; glucose, 0.06; BSA (bovine serum albumin), 15; and (O_2, 95% + CO_2, 5% mixed)/10 min, pH 7.4] containing

(w/v) collagenase type XI (0.1%), BSA (5%) and HEPES [N-(2-hydroxyethyl-piperazine)-N'-(2-ethanesulfonic acid)], 0.6%, Sigma-Aldrich®, St Louis, MO, USA) was injected into the common bile duct of the rat. The swollen pancreas was then quickly excised and incubated at 37°C. Islets were collected with the aid of a stereomicroscope.

A batch of the isolated pancreatic islets was used to study the function of muscarinic and adrenergic receptors by incubation under the cholinergic and adrenergic agents, while another batch was used to quantify the protein expression of the α2AR receptor by western blot analysis.

2.6 Modulation of insulin release by muscarinic and adrenergic drugs

Isolated islets were adapted to a baseline glucose concentration (5.6 mmol/l) during a preincubation period of 60 min in 1 ml of normal Krebs-Ringer solution [(mmol/l): NaCl, 115; $NaHCO_3$, 24; KCl, 1.6; $MgCl6H_2O$, 1; $CaCl_22H_2O$, 1; BSA, 15] and pH 7.4. After preincubation, the solution was discarded, and the islets were incubated with different agonists and antagonists and then diluted in 1 ml of Krebs-Ringer solution for an additional 60 min.

To assess the function of the muscarinic receptor, islets were incubated with 8.3 mmol/l of glucose in the absence or presence of 10 µmol/l of ACh and 10 µmol/l of neostigmine (Neo) to block acetylcholinesterase action and to observe the maximum secretory capacity of pancreatic islets. In addition, islets were incubated with either methoctramine (MTT, 1 µmol/l, an antagonist for the muscarinic receptor M2mAChR) or 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, 100 µmol/l, an antagonist for the muscarinic receptor M3mAChR).

To assess the function of the adrenergic receptor, islets were incubated with 16.7 mmol/l of glucose in the absence or presence of 1 µmol/l of epinephrine (Epi). Then, the islets were incubated with either yohimbine (Yoh, 10 µmol/l, an antagonist for the alpha-2 adrenergic receptor) or propranolol (Pro, 1 µmol/l, an antagonist for the beta-adrenergic receptor) in the presence of 16.7 mmol/l of glucose and 1 µmol/l of Epi. All incubations were collected for further insulin measurement by RIA as described above.

*2.7 Protein expression of the alpha-2 adrenergic receptor (*α2AR)

After isolation, groups of islets were resuspended in RIPA buffer and were lysed by sonication. The lysates were centrifuged at 12,000 rpm for 20 min at 4°C, and total protein content was determined using a BCA™ Protein Assay Kit (Thermo Scientific®, Rockford, IL, USA) and a microplate reader (Multi-Mode Reader, FlexStation® 3 Benchtop, Molecular Devices, Sunnyvale, CA, USA). The samples were treated with Laemmli buffer (w/v: glycerol, 20%; β-mercaptoethanol, 10%; sodium dodecyl sulfate (SDS), 40%; Tris, pH 6.8, 0.5 mol/l; bromophenol blue, 0.5%; and deionized water).

Total protein extracts (30 µg) were resolved by 10% SDS-PAGE. The proteins were then transferred from the gel to a nitrocellulose membrane by the Trans-Blot® Semi-Dry Electrophoretic Transfer Cell (Bio-Rad®, Hercules, CA, USA) and were blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TTBS; Tris-HCl, 1 mol/l; NaCl, 5 mol/l; and Tween-20, 0.05%; v/v) for 90 min with continuous shaking. Membranes were incubated overnight at 4°C with a rabbit polyclonal primary antibody against the alpha-2 adrenergic receptor at a 1:1000 dilution (Sigma-Aldrich®, St. Louis, MO, USA) and were followed by incubation

with peroxidase-conjugated anti-rabbit secondary antibodies at a 1:10000 dilution (Sigma-Aldrich®, St. Louis, MO, USA). Immunoreactive proteins were visualized with ECL (GE Healthcare, Buckinghamshire, UK) and detected by chemiluminescence (ChemiDoc™ XRS System, Bio-Rad®, Hercules, CA, USA).

The bands were quantified by densitometry using ImageJ 1.4 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). β-actin protein content (Santa Cruz Biotechnology®, Santa Cruz, CA, USA; diluted 1:1000 in TTBS) was utilized for normalization (Miranda et al., 2014). Each of the two lanes represents protein expression of α2-AR from a pool of 300 islets, which were collected from 3 different rats. Thus, each of the 2 lanes/group represent a different sample. The experimental average, quantified in the bar graph, is the result of three independent experiments, each with 2 different samples/group. Furthermore, the images were not transformed or adjusted, we utilized raw images for the quantifications.

2.8 Biometrical and biochemical parameters

BW was measured at 60 and 120 days old. At 120 days old, after an overnight fasting, the animals were euthanized and the blood was collected for glycaemia and insulinemia dosages, and the fat pads were removed and weighed. The plasma obtained from the blood samples was stored at -20°C until measured by the methods described above.

2.9 Statistical analysis

All data were subjected to normal distribution analysis by the D'Agostino-Pearson omnibus test. The results are shown as the mean ± the standard error of the

mean (SEM) or as the percentage of variation (data on insulin release in the presence of different drugs). To compare the SO and SYM groups, the data were subjected to repeated measures ANOVA followed by Holm-Sidak post hoc multiple comparisons test; one-way ANOVA followed by Tukey's post hoc test; or Student's t-test. P values less than 0.05 were considered statistically significant. The statistical tests were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

Table 1 shows that the final BW from the SYM group (426.7 ± 4.70) increased 8.5% compared with that from the SO (393.2 ± 4.43), ($p < 0.001$). The SYM group showed an increased accumulation of white adipose tissue by 20.8% and 15.6% (1.51 ± 0.03 and 1.08 ± 0.02) in the retroperitoneal and mesenteric fat pads when we compared with that of SO rats (1.25 ± 0.03 and 0.93 ± 0.04, respectively) ($p < 0.0001$; $p < 0.005$). However, the fasting glycaemia levels were not different between the groups (72.2 ± 2.40 and 75.1 ± 1.81, SO and SYM groups, respectively) ($p > 0.05$). The fasting insulinemia level of the SYM rats (0.30 ± 0.01) decreased by 21% compared with that of the SO (0.38 ± 0.02) ($p < 0.01$). The HOMA-IR index from the SYM rats (1.52 ± 0.07) decreased by 24% compared with that from the SO rats (2.00 ± 0.15), ($p < 0.05$).

3.1 Intravenous glucose tolerance test

At the 5-, 15-, 30- and 45-min time points of the ivGTT, the SYM animals exhibited increased glycaemia; the AUC shows an increase of 15.3% in the glucose concentration (698.9 ± 7.43 and 806.0 ± 9.70, SO and SYM group, respectively) (p <

0.001), as shown in the inset of figure 1A. In contrast, the level of insulinemia was diminished during the 0-, 5- and 15-min ivGTT ($p < 0.001$; $p < 0.001$; $p < 0.01$), which can also be verified in the AUC inset with a reduction of 29.7% (2.52 ± 0.13 and 1.77 ± 0.07, SO and SYM group, respectively) ($p = 0.0005$; figure 1B).

3.2 Functional study of the isolated pancreatic islets

In the first assay, islets from both animal groups were tested in the presence or absence of muscarinic binding. Figure 2A shows that islets from SYM rats, under the action of glucose (8.3 mmol/l) only, secreted 40.9% less insulin than the islets from the SO group (1.22 ± 0.06 and 0.72 ± 0.02, SO and SYM group, respectively) ($p = 0.0002$); moreover, the secretory response was reduced in the presence of ACh (1.80 ± 0.09 and 1.14 ± 0.03, SO and SYM group, respectively), MTT (2.02 ± 0.10 and 1.42 ± 0.04, SO and SYM group, respectively) and 4-DAMP (1.01 ± 0.05 and 0.68 ± 0.02, SO and SYM group, respectively) by 36.7%, 29.7% and 32.6%, respectively, between the SO and SYM groups to the same drug ($p < 0.0001$; $p < 0.0001$; $p < 0.05$). However, in this study, it was necessary to analyse the percentage of variation between each drug, since the SO and SYM groups exhibited two different starting points with glucose (8.3 mmol/l) only. The SYM islets were more responsive to ACh action; the insulinotropic effect of ACh was 58% in islets from SYM rats and 47% in islets from the SO group ($p < 0.05$, figure 2B). When the islets were incubated in the presence of MTT, the SYM animals were approximately 2-fold more responsive to MTT than the SO rats ($p = 0.0008$; figure 2C); however, incubation with 4-DAMP resulted in similar insulin release in both groups (figure 2D).

In the second approach, islets were incubated in the presence or absence of adrenergic agonists or antagonists. As observed in figure 3A, under the action of glucose (16.7 mmol/l) only, islets from SYM rats secreted 43% less insulin than the islets from the SO rats (2.36 ± 0.07 and 1.34 ± 0.05, SO and SYM group, respectively) ($p < 0.0001$); however, we did not observe changes in the presence of Epi (0.95 ± 0.06 and 0.73 ± 0.05, SO and SYM group, respectively), Yoh (1.05 ± 0.07 and 0.75 ± 0.06, SO and SYM group, respectively) or Pro (0.86 ± 0.05 and 0.68 ± 0.05, SO and SYM group, respectively) in the SO or SYM groups. Moreover, in analysing the percentage of variation with each drug (SO and SYM groups evidenced two different starting points with glucose 16.7 mmol/l only), we observed an inhibitory effect of epinephrine 1 μmol/l, resulting in 60% reduction in the insulin release in islets from SO islets rats and 45% in islets from the SYM group ($p < 0.0001$, figure 3B). The incubation with the adrenergic-specific antagonists Yoh and Pro did not result in changes between the groups (figures 3C and 3D).

3.3 Alpha-2 adrenergic receptor expression - α2AR

The protein expression of the α2AR was increased by 43% in pancreatic islets from SYM animals when compared with the islets from SO rats ($p < 0.001$; figure 4).

4. Discussion

The current study shows that the SNS ends that innervate beta cells are essential for metabolism and the maintenance of glucose homeostasis through the control of insulin release. Our findings showed that adrenergic denervation results in imbalanced fat deposition, impaired fasting insulin levels, and increased glucose tolerance. Our data also showed alteration in the function of α2AR in pancreatic

islets. Therefore, the loss of function in pancreatic sympathetic fibres is related to metabolic dysfunctions in adult rats.

Studies have shown that obese animals exhibit a general imbalance in the ANS, which is characterized by high parasympathetic activity and low sympathetic tonus (Barella et al., 2012; Barella et al., 2015; Scomparin et al., 2009). This imbalance is accompanied by several metabolic dysfunctions, including increased body weight and fat deposition, as well as altered glucose metabolism and beta cell function. Here, we show that rats lacking pancreatic sympathetic innervation exhibit alterations in metabolism that are similar to that in obese animals. These results suggest that the sympathetic nerves play a substantial role in glucose homeostasis and that insulin secretion is pivotal. On the other hand, malnourished animals exhibit an ANS imbalance in the opposite pattern, i.e., low parasympathetic and high sympathetic activity, in addition to impaired pancreatic islet function; however; in this case they show an increase in insulin sensitivity (de Oliveira et al., 2011; de Oliveira et al., 2013).

Interestingly, we found a high tissue insulin sensitivity, as shown by the HOMA-IR index. Despite the slight increase in glucose blood levels during the ivGTT, we found a decrease in insulin blood levels in response to glucose load. Taken together, these data demonstrate that the peripheral tissues of SYM rats are more sensitive to insulin than the SO rats; indeed, even with low plasma insulin levels, the blood glucose levels of the animals were controlled after 30 min during the ivGTT. Consistent with previous studies, we demonstrated alterations in the adrenergic system through different experimental models that led to the adaptation in both insulin release and insulin sensitivity. (Asensio et al, 2005; Savontaus et al, 2008; Ruohonen et al, 2012).

Both chemical and genetic ablation of the sympathetic nerves were previously shown to impair the development of the pancreas, compromise insulin release and impair glucose homeostasis, thus contributing to an increased risk of type 2 diabetes. Interestingly, in either 8 days or 24 h after chemical sympathectomy, female and male mice showed a significant decrease in plasma insulin levels (Ahrén and Lundquist, 1981; Trudeau, et al, 1990). In addition, dysfunction of sympathetic activity correlated with age and many pancreatic pathologies, such as type 2 diabetes (ahren et al, 2015; Persson-Sjogren et al, 2005). Furthermore, the reduced insulin levels could be associated with reduced insulin mRNA levels in chemical sympathectomized mice (Kvist-Reimer *et al.*, 2002).

The beginning of the SNS development start even during pancreatic development; moreover, in the peripheral nervous system, the postganglionic sympathetic fibres originate from the celiac and superior mesenteric ganglion (Borden et al, 2013; Kiba, 2004). An interesting study has shown that sympathetic innervation is crucial to coordinate both islet organization and function in the initial stages of life (Borden et al., 2013). Although we did not analyse the islet architecture or cell organization or perform the sympathectomy later in life, our data show some metabolic dysfunctions similar to that exhibited during sympathetic denervation that occurs early in life (Borden et al., 2013). These findings suggest that, even later in life, the sympathetic tonus may play an important role in the maintenance of regulatory function of the pancreatic islets and consequently glucose-insulin homeostasis.

Other mechanisms could also contribute to the decrease in insulin release response in our SYM rat model. In pancreatic islets, the PNS and SNS have antagonistic responses and thus show permanent tonus. However, in certain

physiological states, such as physical exercise, the PNS and SNS are interdependent (Kiba, 2004; Mc Corry, 2007; Rodriguez-Dias & Caicedo, 2014). Sympathetic ablation may have also triggered changes in the activity of the PNS in the pancreatic islets by the responsiveness and/or non-responsiveness of mAChR. SYM animals presented an increased cholinergic response, which can also be observed as an increased insulin release in presence of ACh *in vitro*. In addition, insulin release was less responsive to catecholamines in SYM animals. Regarding these results, we can suggest that increasing the response to ACh and decreasing the response to Epi may be an adaptive mechanism to the low levels of insulin released by SYM rats.

Insulin release is regulated by many factors, such as nutrients, hormones and neuronal inputs from the ANS (Ahrén, 2000; Tang et al, 2014). However, the exact mechanisms of communication between the SNS and PNS in the pancreatic islets are unknown. We have shown that SNS ablation causes changes in insulin release *in vitro* in both adrenergic and cholinergic systems. Muscarinic receptor subfamily can be divided into subtypes that potentiate the glucose-insulin response (M3mAChR and, to a lesser extent, M1mAChR); however, stimulating the subtypes M2mAChR and M4mAChR resulted in the attenuation of the M1mAChR and M3mAChR muscarinic subfamily response, which control muscarinic activity on beta cells. Our results demonstrate an increase in insulin release when M2mAChR was blocked, which suggests that the sympathetic system interacts with the parasympathetic system. Although we have not analysed the protein expression of the M3mAChR receptor, our data suggest that the sympathectomy led to the overexpression of insulinotropic receptors as a compensatory response to the decreased insulin release in SYM rats. On the other hand, it has been shown that the insulin levels in α2AR knockout mice were not normalized after treatment with a muscarinic cholinergic

antagonist *in vivo*, indicating that the main cause altering insulin levels was due to the lack of α2AR (Savontaus et al, 2008).

The SNS plays an important role in the regulation of insulin release by binding α2AR, which inhibits insulin release and βAR, thus potentiating insulin release stimulated by glucose. In our current study, we found an upregulation of α2AR in SYM rats, which could be due to a response to the diminished or absent sympathetic innervation resulting from the sympathectomy. In fact, other experimental studies demonstrated that the ablation of the sympathetic system in different phases of development leads to the sensitization of receptors, which leads to adaptations in both expression and responsiveness (Asensio et al, 2005; Ruohonen et al, 2012).

Studies with chemical sympathectomized rats or transgenic mice that overexpress the α2AR showed normalization of insulin release when they were treated with an adrenergic antagonist (Ahrén et al, 1981; Devedjian et al., 2000). Genetic variations in the alpha-2 adrenergic receptor gene (ADRA-2A) in humans that leads to α2AR overexpression also reduces the ability of pancreatic beta cells to release insulin and increases the risk of type 2 diabetes (Rosengren et al., 2010). Moreover, transgenic mice lacking the βAR (beta-less mice) exhibit obesity at adulthood (Asensio et al., 2005). Thus, impairments in the adrenergic system in both humans and animal experimental models can lead to obesity and metabolic diseases, and the altered expression and/or responsiveness of these receptors are associated with beta cell dysfunctions that can induce the onset of type 2 diabetes.

5. Conclusion

The current study demonstrates the importance of SNS innervation to the pancreatic islets regarding the regulation of insulin release and the relative expression of the α2AR. Our findings demonstrate that the overexpression of α2AR may facilitate the onset of type 2 diabetes. Furthermore, ablation of the SNS leads to alterations in the response to insulin release in the presence of a PNS-related muscarinic agonist; therefore, we suggest that the interplay between the two branches of the ANS is not a simple antagonism as described in the literature; however, the underlying mechanism of this interplay needs further study.

Competing interests

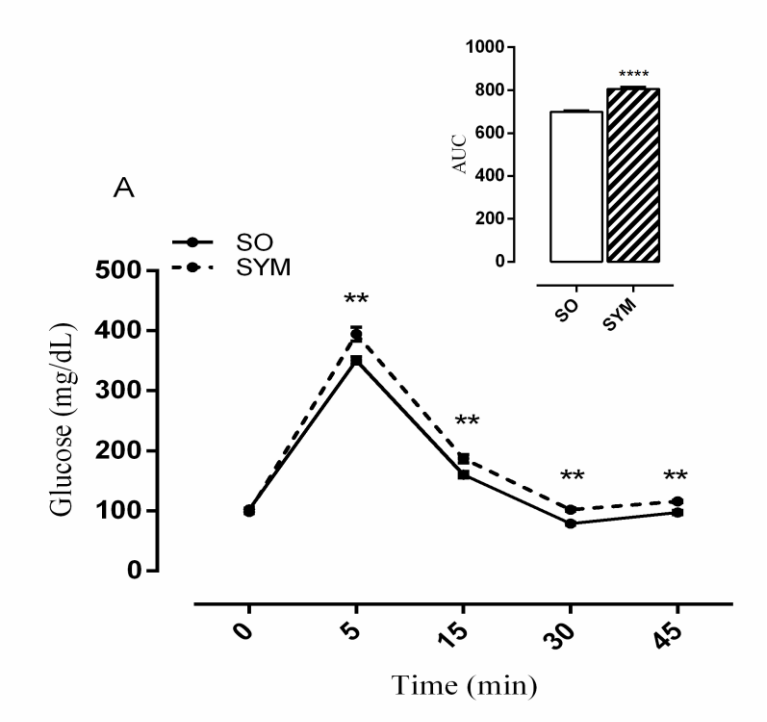
There are no competing interests to be declared.

Table 1. Body weight, Fat pads, Fasting Glycaemia and Insulinemia, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) at 120 days of age

PARAMETERS	SO	SYM	*P* value
Body weight (g) at 60 days of age	245.7 ± 2.45	240.9 ± 1.92	= 0.15
Body weight (g) at 120 days of age	393.2 ± 4.43	426.7 ± 4.70	< 0.001***
Retroperitoneal fat pad (g/100g BW)	1.25 ± 0.03	1.51 ± 0.03	< 0.0001****
Mesenteric fat pad (g/100g BW)	0.93 ± 0.04	1.08 ± 0.02	= 0.005**
Fasting glycaemia (mg/dL)	72.2 ± 2.40	75.1 ± 1.81	= 0.13
Fasting insulinemia (ng/mL)	0.38 ± 0.027	0.30 ± 0.015	= 0.03*
HOMA-IR	2.00 ± 0.15	1.52 ± 0.07	< 0.05*

Data are presented as mean S.E.M. of 11-15 rats from each experimental group. Comparison of SO, sham-operated and SYM, sympathectomized by Student's t-test.

Figure 1



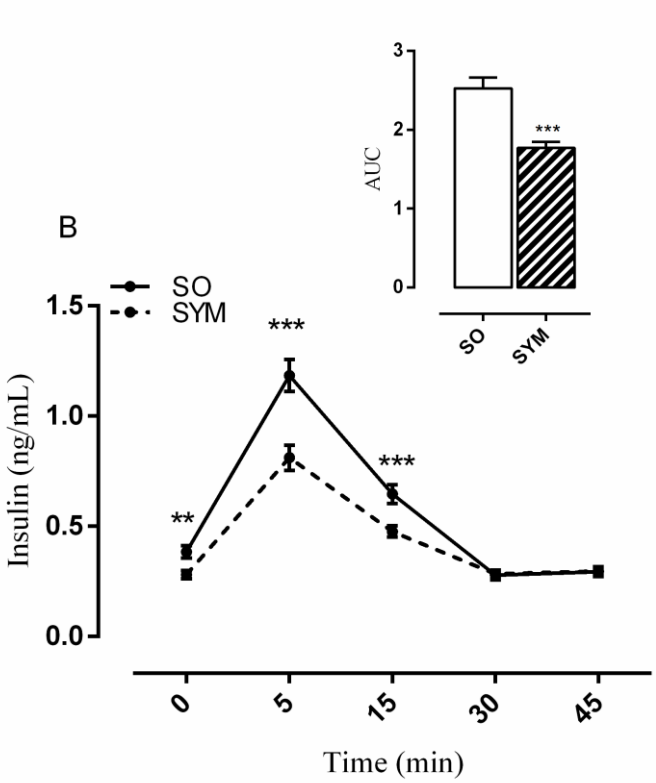


Figure 1. Plasma glucose and insulin concentrations from the intravenous glucose tolerance test (ivGTT). (A) Glycaemia and (B) insulinemia from 15 animals for each group, and the data represent the mean ± SEM. The insert in the upper right panel shows the area under the curve (AUC) calculated from the entire ivGTT. The open bars represent the sham-operated rats, SO, and the filled bars represent the sympathectomized rats, SYM. The asterisk represent the significant difference calculated by Repeated Measures ANOVA and post hoc comparisons by Holm-Sidak's. For the AUC was applied Student's t-test. **p < 0.01; ***p < 0.001; ****p < 0.0001.

Figure 2

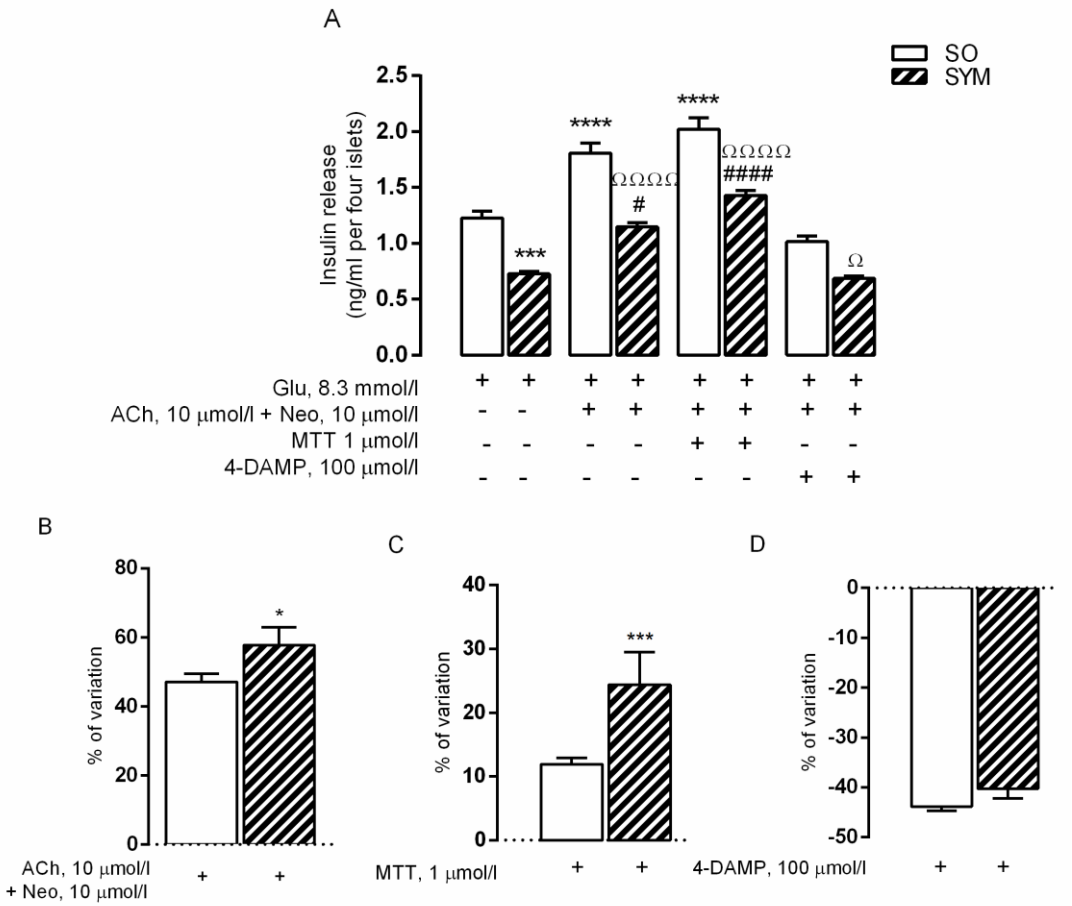


Figure 2. *In vitro* stimulation of isolated pancreatic islets in presence of muscarinic agonist and antagonists. Bars represent mean ± SEM of insulin release of the pancreatic islets from 15 rats from each group. (A) Insulin release stimulated by Glu 8.3 mmol/l and potentiated by ACh 10 µmol/l + Neo 10 µmol/l. (B) Percentage of variation of insulin release potentiated by ACh 10 µmol/l, in presence of Neo 10 µmol; the line from 0 represents the 100% of glucose-induced insulin release. (C) Percentage of variation of insulin release in presence of MTT 1 µmol/l or (D) 4- DAMP 100 µmol/l; the line from 0 represents the stimulation with Glu 8.3 mmol/l and potentiated by ACh 10 µmol/l + Neo 10 µmol/l. Symbols over the bars refer to significance levels: * represent difference between Glu 8.3 mmol/l of sham-operated (SO group) with other drugs; # represent difference between Glu 8.3 mmol/l of sympathectomized (SYM group) with other drugs; Ω represent difference between SO and SYM groups in the presence of the same drug. (A) Analyzed by One-way ANOVA and post hoc comparisons by Tukey. (B, C and D) Were applied Student's t-test. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$; $^{\#}p < 0.05$; $^{\#\#\#\#}p < 0.0001$; $^{\Omega}p < 0.05$; $^{\Omega\Omega\Omega\Omega}p < 0.0001$. ACh, acetylcholine; Glu, glucose; MTT, methoctramine; Neo, neostigmine; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide.

Figure 3

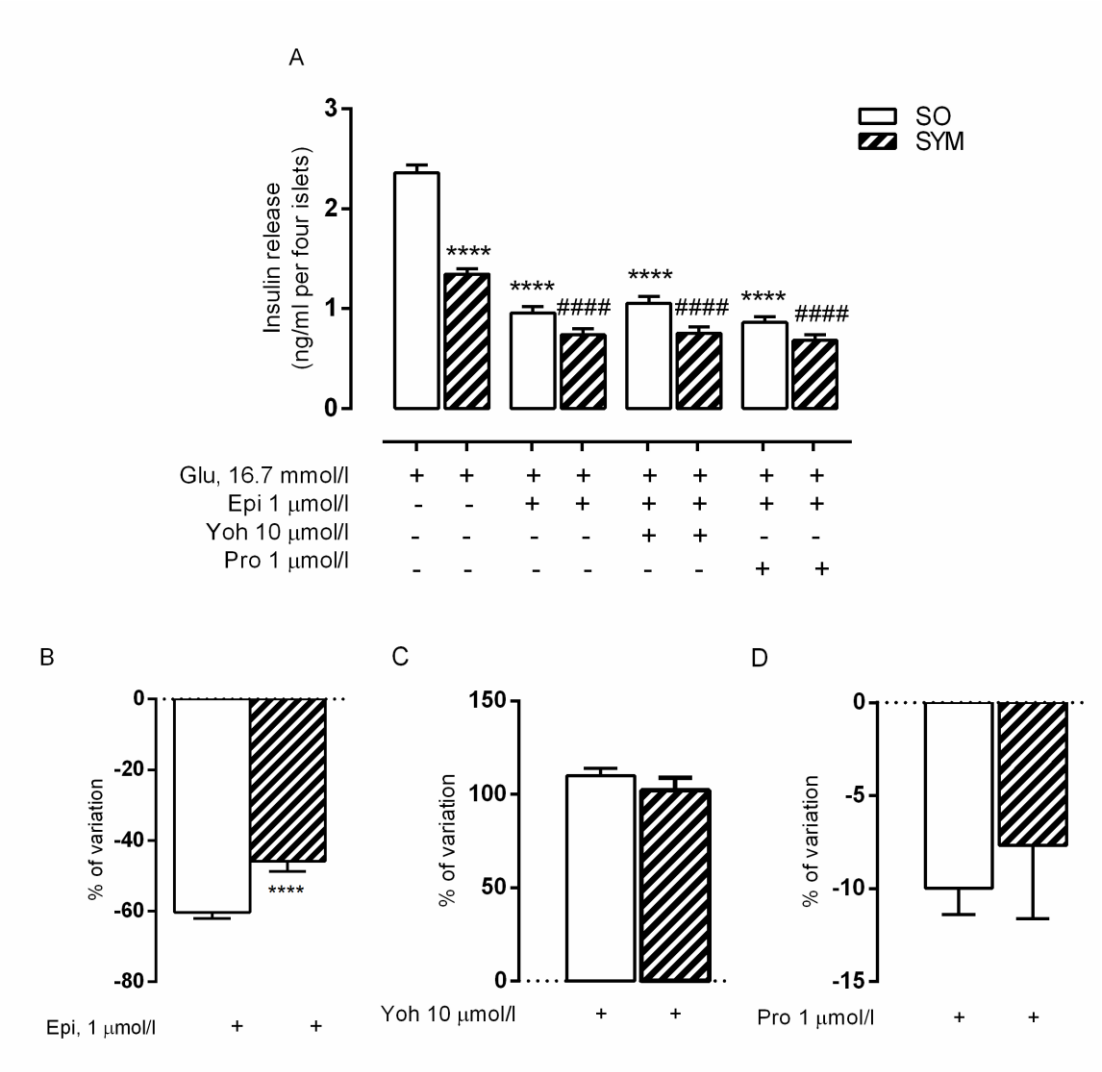


Figure 3. *In vitro* stimulation of isolated pancreatic islets in presence of adrenergic agonist and antagonists. Bars represent mean ± SEM of insulin release of the pancreatic islets from 15 rats from each group. (A) Insulin release stimulated by Glu 16.7 mmol/l and inhibited by Epi 1 µmol/l. (B) Percentage of variation of insulin release inhibited by Epi 1 µmol/l; the line from 0 represents the 100% of glucose-induced insulin release. (C) Percentage of variation of insulin release in presence of Yoh 10 µmol/l or (D) Pro 1 µmol/l; the line from 0 represents the stimulation with Glu 16.7 mmol/l and inhibited by Epi 1 µmol/l. Symbols over the bars refer to significance levels: * represent difference between Glu 16.7 mmol/l of sham-operated (SO group) with other drugs; # represent difference between Glu 16.7 mmol/l of sympathectomized (SYM group) with other drugs. (A) Analyzed by One-way ANOVA and post hoc comparisons by Tukey. (B, C and D) Were applied Student's t-test. ****$p < 0.0001$; $^{\#\#\#\#}p < 0.0001$. Epi, epinephrine; Glu, glucose; Pro, propranolol; Yoh, yohimbine.

Figure 4

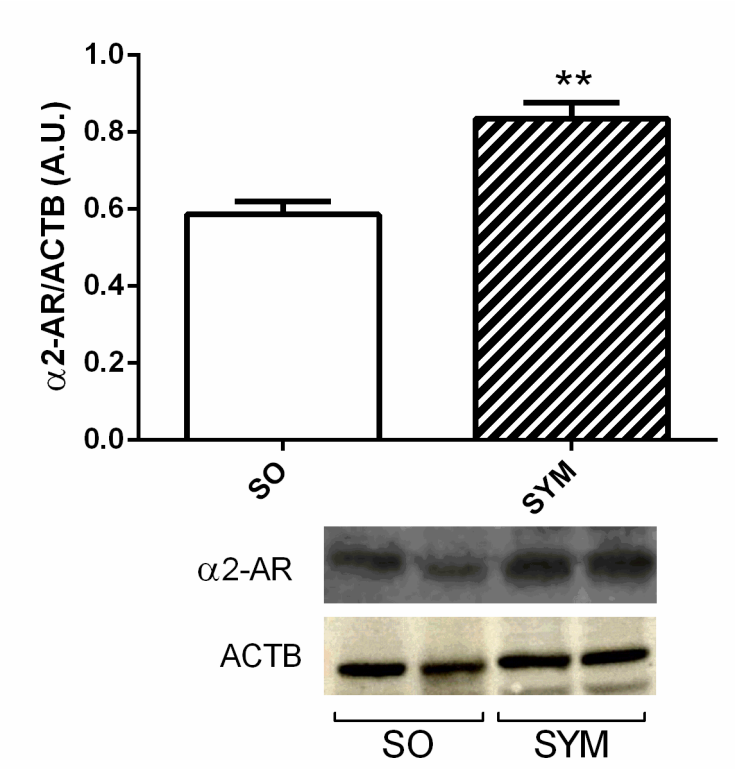


Figure 4. Protein expression of the alpha-2 adrenergic receptor (α2AR) in pancreatic islets. Representative blot are shown below the x-axis. β-actin (ACTB) was used as a control load. Each of the two lanes represents protein expression of α2AR from a pool of 300 islets, which were collected from 3 different rats. Thus, each of the 2 lanes/group represent a different sample. The open bars represent the sham-operated rats, SO, and the filled bars represent the sympathectomized rats, SYM. The data were analyzed by Student's t-test. **$p < 0.0001$.

References

Ahrén et al., 1981. Adrenergic innervation of pancreatic islets and modulation of insulin secretion by the sympatho-adrenal system. Cell Tissue Res 216:15-30

Ahrén, 2009. Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. Nat Rev Drug Discov8, 369-385

Ahrén and Lundquist, 1981. Adrenalectomy and chemical sympathectomy by 6-hydroxydopamine. Effects on basal and stimulated insulin secretion. P flugers Arch 390:17-21

Ahrén et al., 2006. Neuropeptides and the regulation of islet function. Diabetes 55 Suppl 2, S98-S107

Asensio et al., 2005. The lack of beta-adrenoceptors results in enhanced insulin sensitivity in mice exhibiting increased adiposity and glucose intolerance. Diabetes 54, 3490-3495

Barella et al., 2012. Early exposure to a high-fat diet has more drastic consequences on metabolism compared with exposure during adulthood in rats. Horm Metab Res 44, 458-464

Barella et al., 2015.Vagus nerve contributes to metabolic syndrome in high-fat diet-fed young and adult rats. Exp Physiol 100, 57-68

Borden et al., 2013. Sympathetic innervation during development is necessary for pancreatic islet architecture and functional maturation. Cell Rep 4, 287-301

Bray, 1991. Obesity, a disorder of nutrient partitioning: the MONA LISA hypothesis. J Nutr1 21, 1146-1162

Bray and York, 1979. Hypothalamic and genetic obesity in experimental animals: an autonomic and endocrine hypothesis. Physiol Rev 59, 719-809

Burris and Hebrok, 2007. Pancreatic innervation in mouse development and beta-cell regeneration. Neuroscience 150, 592-602

Chen et al., 2012. The worldwide epidemiology of type 2 diabetes mellitus-present and future perspectives. Nat Rev Endocrinol 8, 228-236

Chumasov, 2015. Changes in the pancreatic islets and nervous elements in rats during aging (an immunohistochemical study). Morfologiia 148(6):64-9

Davy and Orr, 2009. Sympathetic nervous system behavior in human obesity. Neurosci Biobehav Rev 33, 116-124

Devedjian et al., 2000. Transgenic mice overexpressing alpha2A-adrenoceptors in pancreatic beta cells show altered regulation of glucose homeostasis. Diabetologia 43, 899-906

Fagerholm et al., 2011. a2-Adrenoceptor Regulation of Blood Glucose Homeostasis. Basic & Clinical Pharmacology & Toxicology, 108, 365–370

Haslam and James, 2005. Obesity. Lancet 366, 1197-1209

Kiba, 2004 Relationships between the autonomic nervous system and the pancreas including regulation of regeneration and apoptosis. Pancreas 29-2

Kvist-Reimer et al., 2002. Effects of chemical sympathectomy by means of 6-hydroxydopamine on insulin secretion and islet morphology in alloxan-diabetic mice. Cell Tissue Res 307, 203-209

Larson et al., 1985. Surgical sympathectomy increases pancreatic polypeptide response to food. Surgery 98, 236-242

Martinez, 2000. Body-weight regulation: causes of obesity. Proc Nutr Soc 59, 337-345

McCorry, 2007. Physiology of the Autonomic Nervous System. Am J Pharm Educ 71(4): 78

Mickley et al., 2006. Acetaminophen self-administered in the drinking water increases the pain threshold of rats (Rattus norvegicus). J Am Assoc Lab Anim Sci 45, 48-54

Miranda et al., 2014. Insulin over secretion in MSG-obese rats is related to alterations in cholinergic muscarinic receptor subtypes in pancreatic islets. Cell Physiol Biochem 33, 1075-1086

de Oliveira et al., 2013. Impaired beta-cell function in the adult offspring of rats fed a protein-restricted diet during lactation is associated with changes in muscarinic acetylcholine receptor subtypes. *Br J Nutr***,** 1-9

de Oliveira JC et al., 2011. Metabolic imprinting by maternal protein malnourishment impairs vagal activity in adult rats. J Neuroendocrinol 23, 148-157

de Souza et al., 2003. Peroxisome proliferator-activated receptor γ coactivator-1-dependent uncoupling protein-2 expression in pancreatic islets of rats: a novel pathway for neural control of insulin secretion. Diabetologia 46:1522–1531

Ribeiro et al., 2016. Acephate exposure during a perinatal life program to type 2 diabetes. Toxicology 30; 372:12-21

Rodriguez-Diaz and Caicedo, 2014. Neural control of the endocrine pancreas. Best Practice & Research Clinical Endocrinology &Metabolism 28(5):745-56

Rosengren et al., 2010. Overexpression of alpha2A-adrenergic receptors contributes to type 2 diabetes. Science 327, 217-220

Ruohonen et al., 2012. Involvement of 2-Adrenoceptor Subtypes A and C in Glucose Homeostasis and Adrenaline-Induced Hyperglycaemia. Neuroendocrinology 96: 51-59

Savontaus et al., 2008. Reduced blood glucose levels, increased insulin levels and improved glucose tolerance in α2A-adrenoceptor knockout mice. European Journal of Pharmacology 359-364

Scomparin et al., 2009. Autonomic activity and glycemic homeostasis are maintained by precocious and low intensity training exercises in MSG-programmed obese mice. Endocrine 36: 510-517

Scott et al., 1981. A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. Diabetologia 21, 470-475

Stein and Colditz, 2004. The epidemic of obesity. J ClinEndocrinolMetab89, 2522-2525

Sun et al., 2015. Pathophysiological Effects of Pancreatic Sympathetic Denervation in Acute Necrotizing Pancreatitis in Dogs Pancreas 44 (7):1083-8

Tang et al., 2014) Imaging of the islet neural network. Diabetes, Obesity and Metabolism.

Trudeau et al., 1990. 6-OHDA sympathectomy and exercise performance in the rat. Arch Int Physiol Biochim. 94:433-417

Wallace et al., 2004. Use and abuse of HOMA modeling. Diabetes Care 27, 1487-1495

Winzell and Ahrén, 2007. G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes. Pharmacol Ther 116, 437-448

Xiong et al., 2010. Effects of Vagotomy, Splanchnic Nerve Lesion, and Fluorocitrate on the Transmission of Acute Hyperosmotic Stress Signals to the Supraoptic Nucleus. Journal of Neuroscience Research 89(2):256-66