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# Role of vesicular monoamine transporter type 2 in rodent insulin secretion and glucose metabolism revealed by its specific antagonist tetrabenazine

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## Abstract

Despite different embryological origins, islet beta-cells and neurons share expression of many genes and display multiple functional similarities. One shared gene product, VMAT2, vesicular monoamine transporter type 2 (also known as SLC18A2), is highly expressed in human beta-cells relative to other cells in the endocrine and exocrine pancreas. Recent reports suggest that the monoamine dopamine is an important paracrine and/or autocrine regulator of insulin release by beta cells. Given the important role of VMAT2 in the economy of monoamines such as dopamine, we investigated the possible role of VMAT2 in insulin secretion and glucose metabolism. Using a VMAT2-specific antagonist, tetrabenazine (TBZ), we studied glucose homeostasis, insulin secretion *in vivo* and ex vivo in cultures of purified rodent islets. During intraperitoneal glucose tolerance tests, control rats showed increased serum insulin concentrations and smaller glucose excursions relative to controls after a single intravenous dose of TBZ. One hour following TBZ administration we observed a significant depletion of total pancreas dopamine. Correspondingly, exogenous L-DOPA reversed the effects of TBZ on glucose clearance in vivo. In *in vitro* studies of rat islets, significantly enhanced glucose-dependent insulin secretion was observed in the presence of dihydrotetrabenazine, the active metabolite of TBZ. Together, these data suggest that VMAT2 regulates *in vivo* glucose homeostasis and insulin production, most likely via its role in vesicular transport and storage of monoamines in beta cells.



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**Keywords**

VMAT2; Insulin; Glucose Homeostasis

## Introduction

D-Glucose, with the synergistic effects of certain amino acids, is the major physiological stimulus for insulin secretion (reviewed in(Henquin 2000)). Net insulin production and glucose homeostasis, however, is regulated by a number of other molecules, including several classical neurotransmitters (Ahren 2000; Brunicardi, et al. 1995) that act directly on beta cells, and indirectly through other target tissues such as liver and skeletal muscle. Many of these molecules function as amplifying agents that have little or no effect by themselves, but enhance the signals generated by the beta cell glucose sensing apparatus(Henquin 2000). For example, during the cephalic phase of insulin release, acetylcholine (ACh) is released via islet parasympathetic innervation. Beta cells express the M3 muscarinic receptor (Duttaroy, et al. 2004) and respond to exogenous ACh with increased inositol phosphate production, which in turn facilitates Na+ ion exit and calcium ion entry. This results in augmented insulin vesicle exocytosis(Barker, et al. 2002). The amino acid glutamate, the major excitatory neurotransmitter in the central nervous system, is present in both alpha - and beta -cells of the endocrine pancreas. Glutamate is stored in glucagon-containing granules(Hayashi, et al. 2003), and is proposed to enhance insulin secretion when it is released into the vicinity of islet cells(Storto, et al. 2006). The presence of metabotropic glutamate receptors on alpha and beta cells themselves suggests the presence of both autocrine and paracrine circuits within islet tissue involved in the regulation of insulin secretion(Brice, et al. 2002).

Other neurotransmitters, such as the monoamines, epinephrine and norepinephine, acting both systemically and via nerve terminals in the vicinity of islets, may act to suppress glucose stimulated insulin secretion by direct interaction with adrenoreceptors expressed (mainly the alpha 2 receptor) on pancreatic beta cells(Ahren 2000; El-Mansoury and Morgan 1998). Beta cells of the endocrine pancreas also express dopamine receptors (D2) and respond to exogenous dopamine with inhibited glucose-stimulated insulin secretion(Ahren and Lundquist 1985; Niswender, et al. 2005; Rubi, et al. 2005; Shankar, et al. 2006). Purified Islet tissue is a source of monoamines, and has been shown to contain 5- hydroxytryptamine, epinephrine, norepinephrine and dopamine(Cegrell 1968; Ekholm, et al. 1971; Hansen and Hedeskov 1977; Lundquist, et al. 1989; Niswender et al. 2005; Wilson, et al. 1974).

Beta cells also have the biosynthetic apparatus to create, dispose of, and store specific neurotransmitters. For example, tyrosine hydroxylase, the enzyme responsible for catalyzing the conversion of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine, L DOPA decarboxylase, responsible for converting L-DOPA to dopamine (Rubi et al. 2005) and Dopamine Beta Hydroxylase, the enzyme that catalyzes the conversion of dopamine to norepinephrine, are present in islet tissue(Borelli, et al. 2003; Iturriza and Thibault 1993). Thus L-DOPA is rapidly converted in islet beta-cells to dopamine (Ahren, et al. 1981; Borelli, et al. 1997). Monoamine oxidase (MAO) is a catabolic enzyme responsible for the oxidative de-amination of monoamines, such as dopamine and catecholamines, and maintains the cellular homeostasis of monoamines. The possible role of MAO in islet function has been studied, (Adeghate and Donath 1991)and MAO has been detected in both alpha and beta cells of pancreatic islet cells, including beta cells(Feldman and Chapman 1975a, 1975b). Interestingly, some MAO inhibitors have been shown to antagonize glucose-induced insulin secretion(Aleyassine and Gardiner 1975). The secretory granules of pancreatic beta cells store substantial amounts of calcium, dopamine and serotonin(Ahren and Lundquist 1985).

In the central nervous system, the storage of monoamine neurotransmitters in secretory organelles is mediated by a vesicular amine transporter. These molecules are expressed as integral membrane proteins of the lipid bilayer of secretory vesicles in neuronal and endocrine cells. An electrochemical gradient provides energy for the vesicular packaging of monoamines, such as dopamine, for later discharge into the synaptic space (reviewed by(Eiden, et al. 2004). Both immunohistochemistry and gene expression studies show that islet tissue and the beta cells of the endocrine pancreas selectively express only one member of the family of vesicular amine transporters, VMAT2 ( vesicular monoamine transporter type 2)(Anlauf, et al. 2003).

Recent studies have shown the feasibility of noninvasive measurements of the amount of VMAT2 in the pancreas as a useful biomarker of beta cell mass both in humans (R. Goland, manuscript submitted) and rodents (Souza, et al. 2006) using [11C] DTBZ (dihydrotetrabenazine) and positron emission tomography, but the possible functional role of VMAT2, as expressed in islet tissue and beta cells, in glucose metabolism has not yet been explored.

As indicated, endogenously synthesized and/or stored monoamine neurotransmitters appear to participate in paracrine regulation of insulin secretion and entrainment of the activity of various cells within islets(Borelli and Gagliardino 2001). Given the important role of vesicular amine transporters in the storage and distribution of monoamine neurotransmitters, we explored the possible of role of VMAT2 in glucose-stimulated insulin secretion using the VMAT2-specific antagonist, TBZ (tetrabenazine)(Scherman, et al. 1983). TBZ acts as a reversible inhibitor of monoamine uptake into granular vesicles of presynaptic neurons (Pettibone, et al. 1984) through its ability to bind to VMAT2 (Scherman 1986) thereby facilitating monoamine degradation by MAO. Monoamine neurotransmitters that are depleted via VMAT2 inhibition by TBZ include serotonin, dopamine, and norepinephine. Administration of TBZ to rats (plasma elimination $t_{½}$ = 2 hrs) reduces dopamine levels by 40%, serotonin by 44%, and norepinephrine by 41% in the brain(Lane, et al. 1976). Although there are other vesicular amine transporters ( e.g VMAT1), tetrabenazine is highly specific for VMAT2, binds to the transporter with a dissociation constant in the nanomolar range, and displays a more than 10,000-fold reduced affinity towards VMAT1(Erickson, et al. 1996; Varoqui and Erickson 1997). Given the known effects of monoamine neurotransmitters on insulin secretion, the expression of VMAT2 by beta cells and the antagonist action of TBZ on monoamine transport, we elected to study glucose metabolism and insulin secretion in vivo and in vitro following treatment with TBZ.

## Materials and methods

### Drugs and reagents

L-epinephrine bitartrate, D-glucose, L-DOPA, sodium citrate were obtained from Sigma Chemicals. All cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA) Tissue culture plates were obtained from Falconware (Becton-Dickinson, Inc., Oxnard, CA). Tetrabenazine and dihydrotetrabenazine were obtained from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program or Tocris Bioscience (Ellisville, Mo). All other chemicals were of the highest commercial quality available.

### Experimental Animals

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Columbia University’s College of Physicians and Surgeons. All experiments were performed in accordance with ‘Principles of laboratory animal care’ (NIH publication no. 85-23, revised 1985). Normal male Lewis rats were obtained from Taconic

(Taconic Inc., Germantown, NY) and were housed under conditions of controlled humidity (55 ± 5%), temperature (23 ± 1 °C), and lighting (light on: 06.00–18.00 h) with free access to standard laboratory Purina rat chow and water. Rats were handled daily to minimize nonspecific stress for more than 7 days before the experiments began. In most experiments it was necessary to measure blood glucose in fasting animals. For these groups, food was removed at the beginning of the light cycle, 6 hours before glucose levels were tested. Fasting rats for longer than eight hours resulted in higher experimental variability. Sixty minutes prior to intraperitoneal glucose tolerance testing (IPGTT), anesthesia of male Lewis rats was induced with isoflurane (3–4% in oxygen) and maintained with 1–2% isoflurane in oxygen. Anaesthetized rats were administered TBZ at the indicated dose by intravenous (i.v.) injection using the penile vein. TBZ or L-DOPA was dissolved in neat sterile DMSO and diluted (always more than 10 fold) in sterile saline. Control rats received injections of vehicle alone (10% DMSO in Saline). Animals were fully recovered for at least 30 minutes before receiving IPGTT. In specified experiments, L-DOPA was injected I.P. at the specified dose at the initiation of IPGTT. Abnormal glucose tolerance was induced by a single intraperitoneal injection of streptozotocin (Sigma Aldrich, St. Louis, Mo) (50 mg/kg) to animals that had been fasted 4 hours to enhance the efficacy of STZ. The STZ solution was prepared fresh by dissolving it in 0.1 M citrate buffer (pH 5.5) and terminally sterile filtered. Control Lewis age and weight matched rats received a 0.5 ml/kg citrate vehicle alone via intraperitoneal injection. The diabetic phenotype induced by STZ was allowed to develop for one week before confirmation by glucose tolerance testing. Animals were considered to have a stable diabetic phenotype after three consecutive measurements of blood glucose with a value of > 300 mg/dl. Animals failing this criterion were not used and euthanized.

### Blood glucose, insulin, glucagon and intraperitoneal glucose tolerance tests measurements

Blood samples were collected between 12:00 noon and 2:00 pm from a superficial blood vessel in the tails of the rats following 6 hr of fasting. The fasting blood glucose (BG) levels of the rats were measured using an Accu-Check blood glucose monitoring system (Roche Diagnostics, Sommerville, NJ). Intraperitoneal glucose tolerance tests (IPGTT) were performed in 6 hour fasting un-anaesthetized animals as previously described (Weksler-Zangen, et al. 2001). Briefly, after baseline BG measurements, animals received an intraperitoneal (i.p.) injection of 2 gm glucose/kg body weight. To minimize stress during the procedure, rats were handled by the same operator during acclimatization and later during weighing and IPGTT. Blood samples (50 μl or 150 ul) were collected at baseline and then again 15 30, 60, 90, and 120 minutes following i.p. glucose. BG concentrations were measured immediately on these samples and the remainder processed. Plasma was immediately separated by centrifugation at 3000 g for 15 min and then stored at - 80 °C until analysis. Insulin and glucagon concentration measurements in rat plasma were performed by ELISA as per the manufacturer's instructions using kits from Linco Research Inc (St. Charles, MI) and Alpco Diagnostics (Salem, NH), respectively. To validate the test, saline injections were performed by the same method. During this experiment, glucose concentration did not differ from baseline at each time point (data not shown). The area under the insulin, glucagon and IPGTT glucose concentration × time curve (AUCIPGTT) was calculated by the trapezoidal rule.

### Islet tissue and glucose stimulated insulin secretion

Rat pancreas digestion, islet isolations, and static insulin secretion assays were performed as previously described (Niswender et al. 2005; Sweet, et al. 2004; Sweet and Gilbert 2006) Purified islets were cultured in RPMI 1640 culture media with 10% fetal bovine serum at 37° C in humidified air (5% CO2) for 18 to 24 hours. Assessment of Insulin secretion in static media was carried out as follows. Islets were handpicked twice into a Petri dishes containing KRB buffer (with 3 mM glucose and 0.1% BSA) and pre-incubated for 60 min (37°C and 5% CO2). Subsequently, batches of 100 islets (in quadruplicate) were transferred into 96-well

plates containing 200 µl of KRB with either 3 or 20 mM glucose, with or without 100 nM DTBZ and incubated for 60 min. The supernatant was removed and the insulin was measured using ELISA (ALPCO, Windham, NH).

## Dopamine measurements

Anaesthetized rats received an intravenous injection of TBZ and were sacrificed one hour later. Euthanasia was performed by exsanguination of the anesthsized animal. Brain and pancreas were harvested as quickly as possible and frozen at −80 °C until use. Frozen tissue was pulverized in a liquid nitrogen-cooled mortar and extracted in 0.01 N HCL. The tissue extract was centrifuged at 10,000 x g at 4 C to remove debris and the total protein was estimated by reading the absorbance at 280 nm. The concentration of dopamine in the extract was estimated using an ELISA kit from Rocky Mountain Diagnostics (Colorado Springs, Co) as per the manufacturer's instructions and normalized to the extract protein concentration.

## Quantitation of VMAT2 mRNA in pancreata and islets of Lewis rats

Harvesting of pancreata was performed as follows; anesthsized rats were opened with a midline incision and the liver stomach and small intestines reflected to expose the pancreas. The cavity was then bathed with 10 ml of of a 1:1 solution PBS 1x and RNAlater (Ambion, Austin, TX). The pancreas was dissected and transferred transferred to a 50 ml Polypropylene tube containing 6 ml of fresh RNAlater solution and – if not immediately processed -stored at −80 °C. After thawing, the entire pancreas was transferred into 1 ml of QIAzol (QIAGEN, Valencia, CA) / 100mgr of tissue and homogenized. In the indicated experiments, purified and hand picked rat islets (about 500) were transferred directly to QIAzol. Total RNA, either from pancreata or purified islets, was purified using both the RNeasy Mini Kit and the RNase-Free DNase Set from QIAGEN. All RNA extractions were performed using RNase- / DNase- free laboratory ware. RNA was quantified and assessed for purity by electrophoresis on a 1.6% agarose gel and UV spectrophotometry. Tissue processing, RNA extraction, and qRT-PCR assay set up were performed in separate designated laboratory areas to prevent cross-contamination. All reverse transcriptase reactions were performed using the PowerScript RT System from CLONTECH (Palo Alto, CA) with random-priming. The qPCR assays were performed using $1/20^{th}$ of the volume of cDNA obtained from 5 µg of total RNA for pancreas and 0.5 µg for purified islets. The QuantiTect SYBR Green PCR Kit (Invitrogen) was used to perform all the reactions in presence of 0.2 µM primers, in a total volume of 25 µl. Samples were amplified with a precycling hold at 95 °C for 15 min, followed by 36 cycles of denaturation at 95 °C for 15 secs, annealing at 58 °C for 20 s, and extension at 72 °C for 20 s. qRT-PCR reagent controls (reagent alone without RNA or cDNA) were included in all the assays. Each assay was run in triplicates and repeated at least twice to verify the results, and the mean copy number was used for analysis. The standard deviation between assays was not significant (5%) in all the experiments. The relative amount of specific transcripts was calculated as previously described (Maffei, et al. 2004). To correct for sample to sample variations in qRT-PCR efficiency and errors in sample quantitation, the level of both GAPDH and 18 S expression was tested for use in normalization of transcript levels. In these experiments no significant differences were found between normalization by GAPDH mRNA levels or normalization by 18s RNA levels. The oligonucleotides were synthesized by Invitrogen. The primer sequences are as follows: 5'- CGC AAA CTG ATC CTG TTC AT -3' (**VT2-2 F**) and 5'- AGA AGA TGC TTT CGG AGG TG-3' (**VT2-2 R**) 5'- AAC GGA TTT GGC CGT ATC GGA C-3' (**rGAPDH-F**) and 5'- TCG CTC CTG GAA GAT GGT GAT G-3' (**rGAPDH-R**); 5'- TTS GAA CGT CTG CCC TAT CAA-3' (**r18S- F**) and 5'-CAA TTA CAG GGC CTC GAA AG-3' (**r18S R**). The relative amounts of mRNA, were calculated by the comparative cycle threshold (CT) method described by Livak and Schmittgen (Livak and Schmittgen 2001).

### Quantitation of VMAT2 and preproinsulin protein in pancreas lysates by western blot

Western blot analysis was conducted on pancreas tissue obtained from control and diabetic STZ-treated rats using standard procedures. Briefly, sample tissues were flash frozen in liquid nitrogen and ground to a fine powder while frozen. Powdered proteins were solubilized in 1× PBS; 1% Nonidet P-40; 0.5% sodium deoxycholate and 0.1% SDS. A complete cocktail of mammalian protease inhibitors (Sigma-Aldrich, St. Louis, MO), at high concentration, was added immediately prior to sample preparation. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad Inc., Hercules, CA) with bovine serum albumin standards and following the manufacturers recommendations. Solubilized proteins were diluted in Laemmli sample buffer and incubated at 100 °C for 1 min. Protein separation was conducted using the Bio-Rad Lab Mini-gel electrophoresis system on 15% Acrylamide/Bis gels. Proteins were then transferred onto Immobilon-PVDF membranes using the same system. Membranes were prepared for immunoblotting by washing in TTBS (10 mM Tris-Glycine, pH 8.0, 0.15 M NaCl, with 0.05% (w/v) Tween-20). Membranes were then blocked in TTBS plus 5% (w/v) non-fat dry milk. The membranes were separated into high (> 15kD) and low MW ( <15 kd) ranges. Membranes were probed for specific proteins by overnight incubation with either a 1:1,000 dilution of rabbit anti-VMAT2 primary antibody (Chemicon International, Temecula, CA) or a 1:400 dilution anti-insulin primary antibody (Abcam, Cambridge, MA). The membranes were then washed three times in TTBS and developed with 1:5000 dilution of either donkey anti-rabbit Igs or sheep anti-mouse Igs conjugated to horseradish peroxidase (Amersham Bioscience, Pittsburgh, PA) . After one hour, the membranes were washed in TTBS and a chemiluminescent substrate solution was added (Immobilon Western Solution (Millpore, Bedford, MA) . Membranes were then used to expose Bio-Max film (Eastman Kodakm Rochester, NY).

### Statistical analysis

All results are presented as means ± S.E.M., or as indicated in the text. Students T testing was performed for assessing statistical significance of differences. All p values are 2-tailed.

## Results

### Glucose tolerance in Adult Lewis rats is improved by TBZ

Older heavier Lewis rats display glucose intolerance relative to younger animals during an IPGTT.(Natalucci, et al. 2003; Wang, et al. 1997). To explore the role of VMAT2 in insulin secretion, and to better demonstrate the possible value of VMAT2 as a potential therapeutic target, we selected older male Lewis rats (300–500 gms, > 11 weeks of age) for IPGTT testing. For this study only rats with vehicle-alone AUC IPGTT values greater than 10 g/dL x min were used. We selected a doses of tetrabenazine approximately three to ten fold higher than the equivalent doses currently used in humans to treat movement disorders(Kenney and Jankovic 2006). Following TBZ administration (about 1 hr), but before glucose challenge, we did not find reproducible differences in the baseline fasting glucose concentrations of control animals (data not shown). Following tetrabenazine treatment and glucose challenge, however, we found a significant change in the shape of the glucose disposition curve during IPGTT (Figure 1, top panel).

Comparison of the areas under the curve during IPGTT revealed that TBZ reduced the glucose excursion by approximately 35% at 2.25 ug/ gm body weight (Figure 1, bottom panel). This dose represented a maxima of the glucose tolerance enhancing effects of TBZ; at doses lower than that 0.3 mg/ Kg the effects of TBZ became undetectable by this assay, and at doses higher than 5.0 mg/Kg, the AUC IPGTT became increasingly variable, often surpassing that of control levels. Chronic administration of TBZ at approximately 0.1 mg/Kg body weight for five days suppressed the AUC IPGTT in a fashion similar to the single high dose (data not shown).

### TBZ depletes total pancreatic dopamine and L-DOPA reverses effects of TBZ

Dopamine is a well-known substrate of VMAT2-mediated vesicular transport (Howell, et al. 1994) and one of the main reported actions of tetrabenazine is the depletion of dopamine in brain tissue(Kenney and Jankovic 2006). To explore the possible role of dopamine in mediating the in vivo glucose tolerance enhancing effects of tetrabenazine, we examined the effects of tetrabenazine on the concentration of dopamine in both the pancreas and brain. One hour after injection of tetrabenazine, the dopamine content of both tissues was significantly reduced (Figure 2). As islets compose only about 2% of the pancreas, the marked effects of TBZ on total pancreatic dopamine content is likely to reflect dopamine depletion in non islet pancreatic tissue elements as well. We next repeated the IPGTT experiments with TBZ. In these experiments, however, L-DOPA, the metabolic precursor of dopamine or a vehicle control, was administered one hour following TBZ and concurrent with glucose. We found that L-DOPA (6.0 mg/Kg, I.P.) was able to reverse the effects of TBZ, increasing the AUC IPGTT to slightly below control levels, (Figure 1, bottom panel).

### TBZ enhances in vivo glucose-dependent insulin secretion

We next tested the hypothesis that the smaller glucose excursions in IPGTT seen after administration of TBZ were due to increased insulin concentrations in the plasma following glucose stimulation. We measured blood glucose, plasma insulin and glucagon concentrations in blood samples obtained during IPGTT (**Figure 3, panels A-D**). We found that both area under the curve (AUC) insulin and glucagon (GCG) measurements were changed by administration of TBZ, Plasma insulin amounts were significantly greater following a single dose of TBZ or chronic low doses of TBZ (0.1 mg/Kg body weight/day x 5 days) and glucose challenge relative to the vehicle-treated controls. In addition, we found that I.V. dopamine, given at the same time as glucose, partially blocked the insulin enhancing effects of TBZ(Figure 3, panel A). The AUC plasma glucagon measurements were lower relative to controls following i.v. TBZ administration and glucose challenge(Figure 3, panel B and D). The change in AUC glucagon however was less than change in AUC insulin. In STZ treated rats who maintained glucose dependant insulin secretion, TBZ (1.5 mg/Kg) increased the AUC insulin measurement by approximately 50% – 80% and decreased AUC IPGTT (data not shown)

### TBZ enhances in vitro glucose-dependent insulin secretion in purified rat islets

Since VMAT2 is located throughout the CNS and glucose homeostasis is regulated by the autonomic nervous system, a critical question in this study was whether TBZ was acting locally in islets. We next tested whether the VMAT2 antagonist DTBZ, the direct and active metabolite of TBZ, could enhance insulin secretion in purified rat islets tested *in vivo*. The islets were incubated in high and low glucose media with and without dihydrotetrabenazine (DTBZ). Insulin secretion increased ten fold in response to glucose, and was significantly further enhanced by DTBZ two to three fold ($p < 0.05$) (Figure 4). At low glucose, an increase in insulin secretion mediated by DTBZ was not statistically significant.

### VMAT2 is expressed in rodent islets and beta cells

As opposed to VMAT2 expressed by human beta cells (Anlauf et al. 2003), the presence of VMAT2 in rodent islets cannot be detected by immunohistochemistry using currently available commercial antisera. . To demonstrate that VMAT2 is associated with rat islets,, we performed the following series of experiments. First, total RNA was prepared from brain, purified islets obtained from rodent pancreata, and total pancreas . The total RNA was reverse transcribed and amplified with specific primers for rat VMAT2. We were able to amplify and sequence a 175 bp cDNA fragment of the length and structure expected from the published sequence of rat VMAT2 (Erickson, et al. 1992) (Figure 5 panel A). Total RNA from brain was used as a positive control ( Panel A, lane 1). Comparision of the strength of the signal obtained from

islets versus total pancreas showed that VMAT2 RNA was enriched > 20 fold in islets relative to total pancreas (Figure 5 panel A, lane 2 versus lane 3). In the absence of the reverse transcription reaction, no PCR product was found (Figure 5 panel A, lane 4). Within the pancreas, insulin producing beta cells uniquely express the GLUT2 transporter. The toxin streptozotocin selectively targets and destroys beta cells following transport by GLUT2 (Szkudelski 2001)(Elsner, et al. 2000). To demonstrate that VMAT2 is associated with beta cells of the endocrine pancreas, we took advantage of the selective beta cell toxicity of streptozotocin. We prepared total RNA from pancreata obtained from four control rats and four streptozotocin-induced diabetic rats. Quantitation of VMAT2 message by real time PCR showed that treatment with streptozotocin significantly reduced the amount of VMAT2 in diabetic pancreata relative to control pancreata 84 to 92 % (99.9% CI) (Figure 5, Panel B). When protein lysates were prepared from pancreata obtained from control rats and streptozotocin-induced diabetic rats, separated by SDS-PAGE, transferred to membranes and then probed with VMAT2 antibodies, the loss of VMAT2 protein, as well as preproinsulin protein, following STZ treatment was visible by the loss of the western blotting signal (Figure 5, Panel C)

## Discussion

In this report we provide evidence that VMAT2 expressed in beta cells of the endocrine pancreas plays a role in the regulation of insulin production and glucose homeostasis *in vivo*. We further provide evidence that the glucose tolerance enhancing effects of TBZ is mediated by the depletion of dopamine following the antagonism of VMAT2. Our studies focused on dopamine as the most likely intermediate mediator of the effects of TBZ, although it is not ruled out that other monoamines, such as serotonin, etc., also play a role in the observed in vivo effects of TBZ.

Several previous studies have demonstrated a link between insulin secretion and dopamine. For example, treatment of Parkinson's patients with dopamine precursor, L-DOPA, reduces insulin secretion in glucose tolerance tests(Rosati, et al. 1976). In rodent experiments, i.v. administration of L-DOPA inhibits glucose-stimulated insulin secretion (Ericson, et al. 1977; Zern, et al. 1980). In culture, analogues of dopamine inhibit glucose-stimulated insulin release by purified islets(Arneric, et al. 1984). Most recently, Rubi et al (Rubi et al. 2005) demonstrated that mouse beta cells (INS-1E cells), as well as purified rat and human islets, express the dopamine D2 receptor. In these cells and tissues, the D2 receptor was shown to colocalize with insulin in secretory granules in a pattern similar to the colocalization of VMAT2 and insulin (Anlauf et al. 2003). Both dopamine and the D2-like receptor agonist, quinpirole, inhibited glucose-stimulated insulin secretion when tested in primary rat beta cells, and rat, mouse and human pancreatic islets.

Together with the studies of Rubi et al (Rubi et al. 2005) and others (Brodoff and Kagan 1972) the following model for the role of VMAT2 in islet function can be proposed. Dopamine produced locally in the beta cell cytoplasm is normally transported and stored in insulin-containing vesicles. In the presence of tetrabenazine, the vesicular storage of dopamine declines. Under normal glucose-stimulated insulin secretion, dopamine is co-released with insulin and acts either in an autocrine or paracrine fashion to limit glucose-stimulated insulin secretion by other local beta cells. In the presence of tetrabenazine, this negative feedback loop is not present and less dopamine is released with insulin and other local beta cells remain uninhibited by dopamine.

Currently, arginine pulse stimulation of insulin secretion is a gold standard measurement for evaluating functional beta cell mass. Our preliminary studies with TBZ suggest that more detailed glucose clamp and insulin secretion measurements should be performed and we are

evaluating whether inhibition of VMAT2 might further improve the hyperclycemic clamp technique applied to evaluating beta cell mass. Although our studies are incomplete, we find that repeated low doses of TBZ may also be active in reducing glucose excursions. In other unrelated studies we find some evidence from PET studies that DTBZ can accumulating in the pancreas. Together these data suggest that chronic low doses of TBZ may result in antagonism of VMAT2.. Lastly, our observations must be interpreted carefully. Tetrabenazine has been used to treat movement disorders for over thirty years (Kenney and Jankovic 2006) and effects on glucose homeostasis have not been reported. Nevertheless our findings suggest that VMAT2 plays a role in glucose homeostasis and could be a therapeutic target in diabetes.

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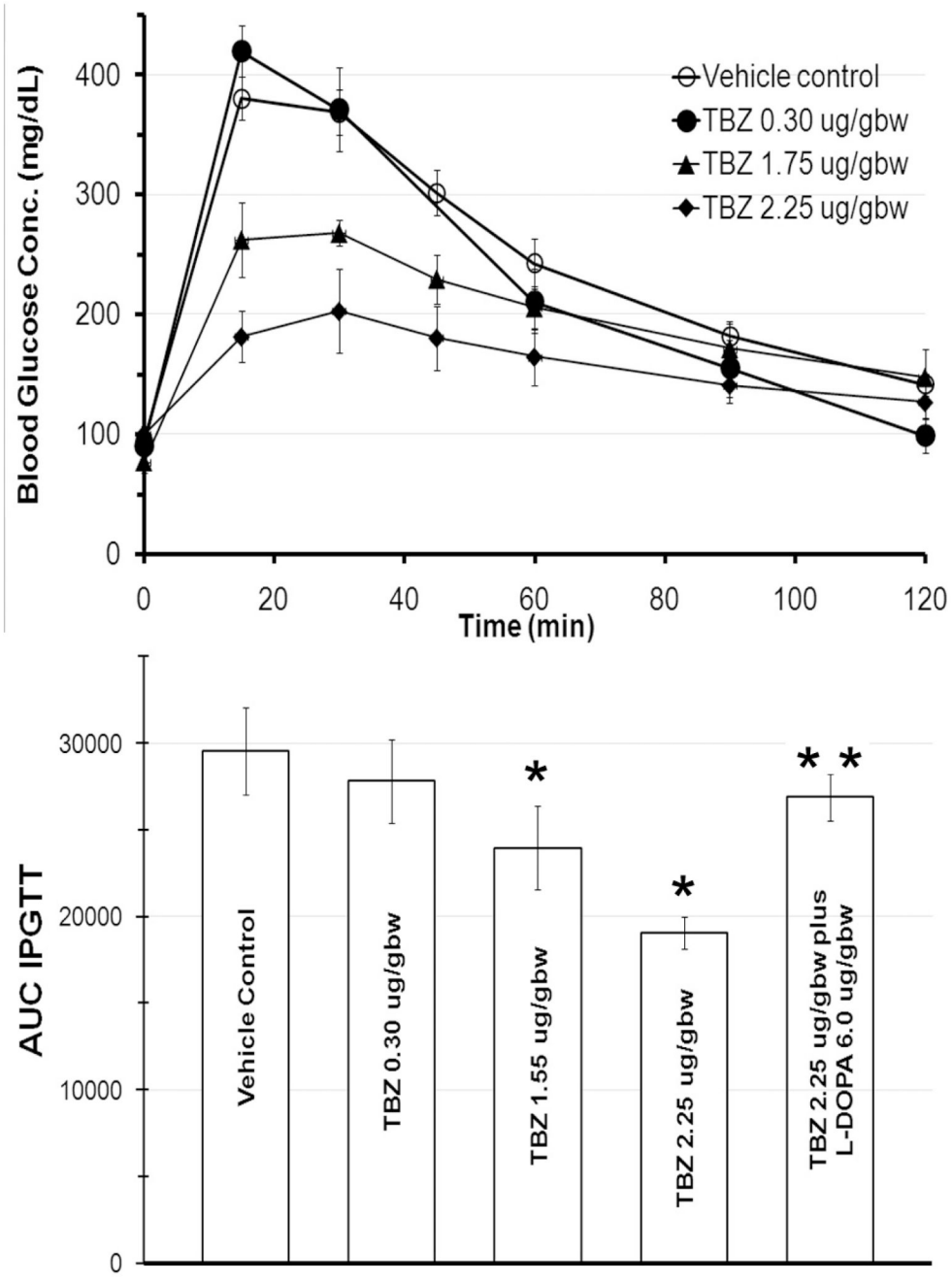


**Figure 1. Tetrabenazine reduces the blood glucose excursion during IPGTT**

*Top Panel*. Blood glucose values during IPGTT of Lewis rats treated with vehicle alone (open symbol) or with tetrabenazine at the indicated doses Error bars indicate the SEM (n=25) at the indicated dose (closed symbols). *Bottom panel*. Results presented as AUC IPGTT. The asterisk indicates AUC IPGTT for vehicle controls (n=25) was significantly higher than the AUC IPGTT of the same TBZ treated animals (n=25) ($p < 0.05$). The double asterisk indicates that the AUC IPGTT following treatment with TBZ and L-DOPA was significantly different than that of TBZ alone (n=6) Error bars represent S.E.M..

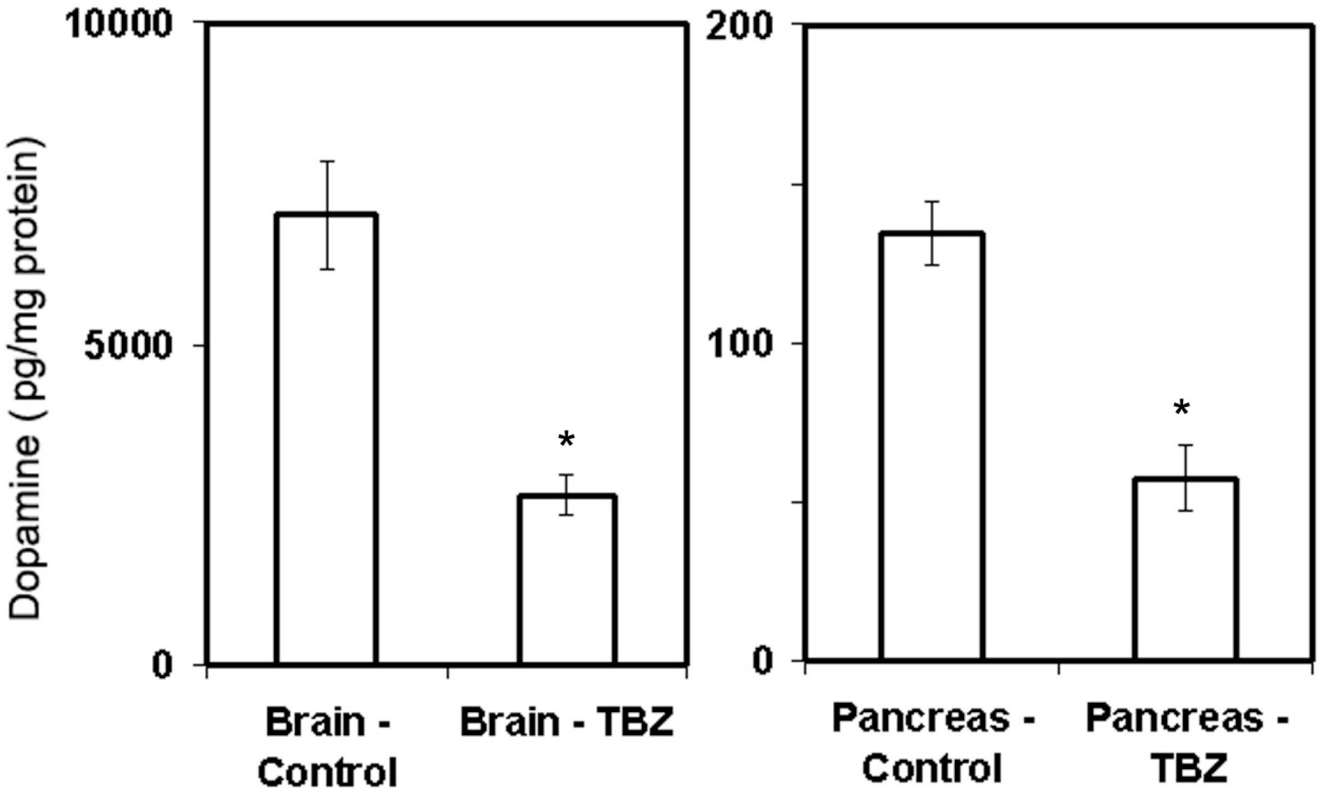


**Figure 2. TBZ reduces the dopamine content of brain and pancreas tissue**

Tetrabenazine at 1.5 mg / Kg body weight was administered i.v. to Lewis rats. One hour later, the animals were euthanized and the brains and pancreata harvested and extracted in buffer. The dopamine concentration in the extract was determined by ELISA and normalized to the total protein content. The error bars represent the S.E.M. from measurements of three TBZ treated and three control Lewis rats. An asterisk represents a significant difference ( $p < 0.05$) from control..

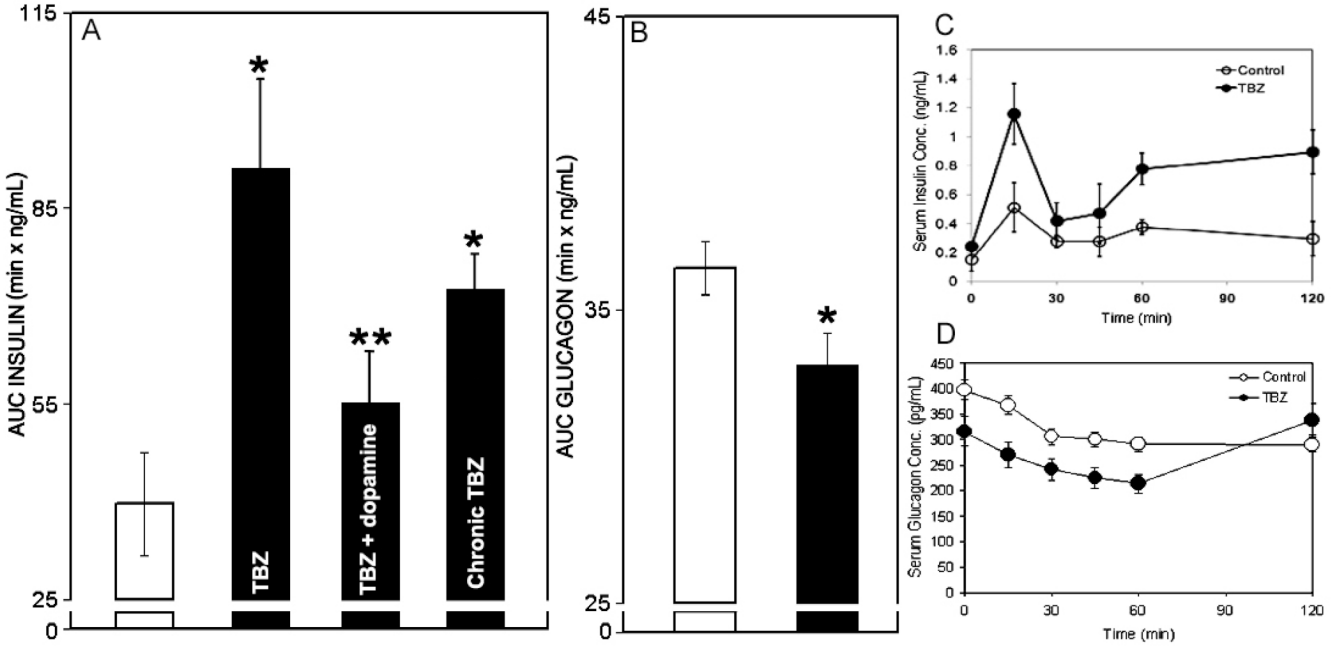


**Figure 3. Tetrabenazine alters glucose stimulated insulin and glucagon secretion *in vivo***

Plasma insulin (panel A and C) and glucagon concentrations (panel B and D) were measured during IPGTT of Lewis rats (>11 weeks old) (n=6) treated with vehicle alone (open columns and circles). One week later, a second IPGTT was performed with TBZ (2.25 mg/Kg body weight)(filled columns and circles), TBZ (2.25 mg/Kg body weight) plus dopamine (6.0 mg/Kg), or following five daily injections TBZ (0.3 mg/Kg body weight). An asterisk represents a significant difference ($p < 0.05$) from control.

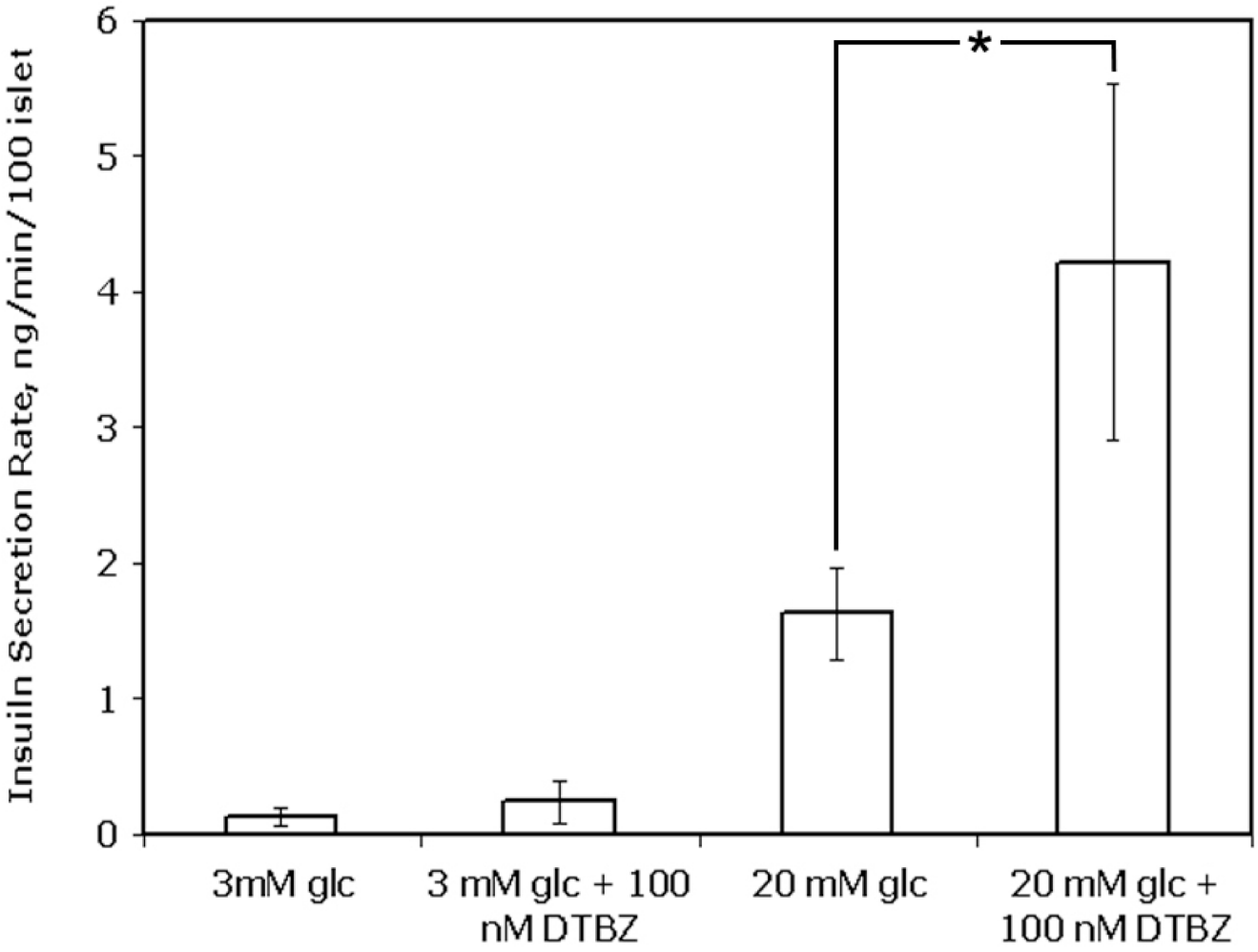


**Figure 4. DTBZ enhances Glucose Stimulated Insulin secretion in rat islets *ex vivo***

Hand picked purified islets were cultured in high or low glucose containing media with and without DTBZ. Serial Insulin concentration measurements in the supernatant were performed and the means and S.E.M. calculated. An asterisk represents a significant difference ( $p < 0.05$) from control.

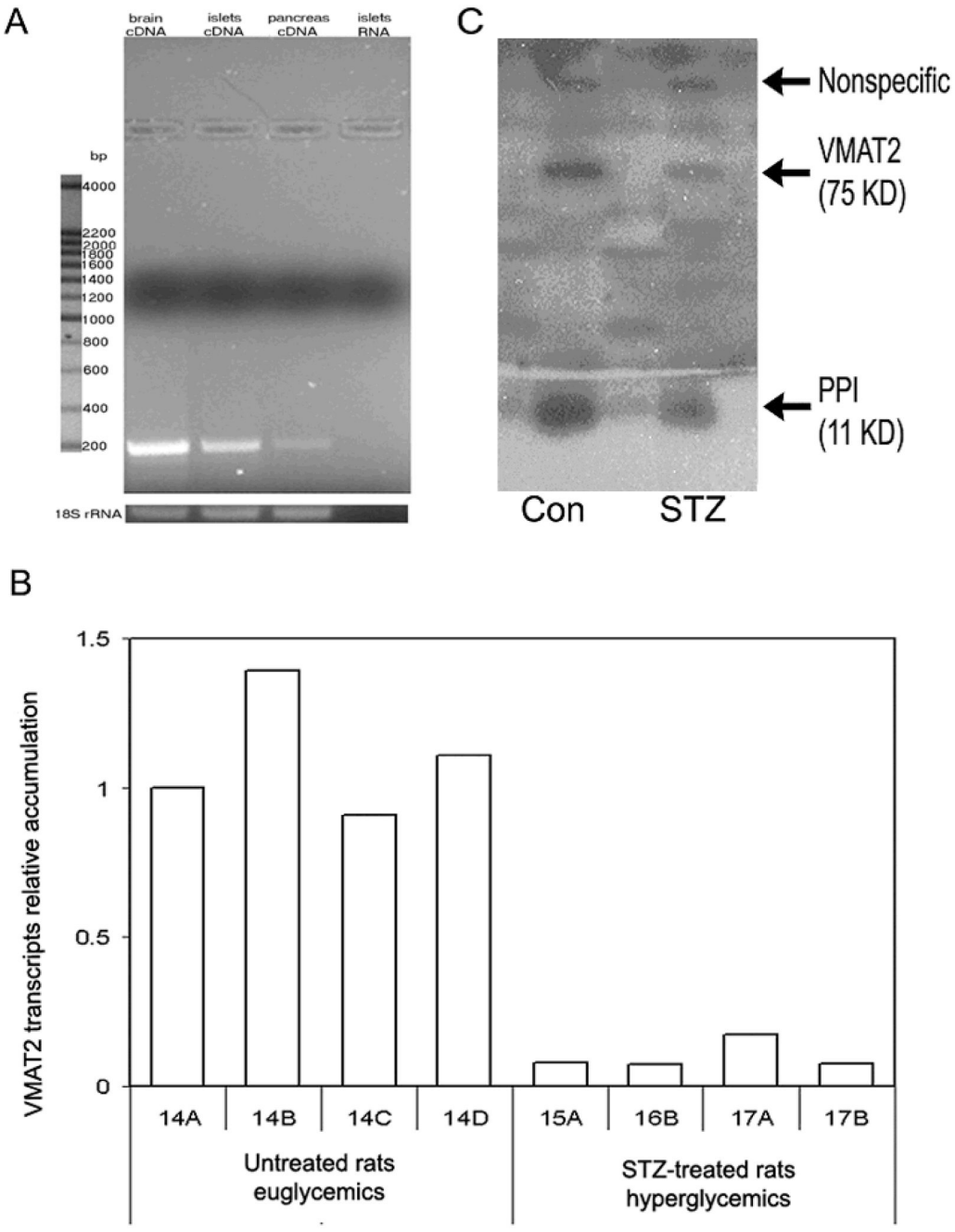


**Figure 5. VMAT2 mRNA and protein in islets and pancreas of control and streptozotocin treated Lewis rats**

Panel A, products of the qRT-PCR assay on total RNA from: brain, purified islets and total pancreas using VMAT2 primers that specifically amplify a 175 bp fragment. Untranscribed RNA from purified islets is used as a control.and GelPilot 200 bp ladder, as the molecular weight standard. Panel B, Relative accumulation of VMAT2 mRNA in pancreata of control and streptozotocin-induced diabetic Lewis rats. The average accumulation of VMAT2 mRNA in streptozotocin-treated rodents was approximately eight-fold lower than the average accumulation in untreated pancreata ($p<0.005$) Panel C, Western blot analysis of VMAT2

expression in protein lysates prepared from control and streptozotocin-induced diabetic Lewis rats.